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ASCITES TUMORS AS TOOLS IN QUANTITATIVE ONCOLOGY

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Editor

ROY WALDO MINER[†]

ASCITES TUMORS AS TOOLS IN QUANTITATIVE ONCOLOGY*

Conference Chairman and Consulting Editor

THEODORE S. HAUSCHKA

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INTRODUCTORY REMARKS

By Hilary Koprowski
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The pattern of development of early investigations of ascites tumors, as well as of many other scientific inquiries, may be compared to the structure of some of the musical masterpieces of the 19th century that were composed as improvisations. The beginning of such compositions usually consists of tentative fragments, breaking off abruptly and alternating with mutually unrelated incomplete phrases. Suddenly a proper tune emerges, but it wanders away.

The discovery of Lowenthal and Jahn (modeled after Hesse's earlier finding), that a solid tumor could be adapted to free growth in the peritoneal cavity, can be considered to be the proper tune that "wandered away." Application of this finding did not occur for two decades. Moreover, we do not know today which of several solid neoplasms was actually transformed into the ascites tumor that now bears the name "Ehrlich."

Returning to the musical parallel, in some compositions—after the proper tune has wandered away—there is often a flourish of rapid, cohesive sequences of the principal theme followed by logical variations. This type of development in the study of ascites tumors was initiated six or seven years ago by the first contributor to this monograph, and was carried on in many directions by several able groups of scientists. Evidence of the remarkable progress of endeavors in this field is the fact that a full two-day conference has been organized on this subject, with participants from all over the world.

It is obvious that the participating scientists do not think they have done away with other tools in mammalian cell research through their investigations of ascites tumors. But the words of Goethe spoken about Faust may well be remembered, "The solution of every problem gives rise to a new problem that needs to be solved."

Part I. Growth and Population Analysis

CONVERSION OF SOLID NEOPLASMS INTO ASCITES TUMORS*

By George Klein and Eva Klein
Wallenberg Laboratory, Institute for Cell Research and Genetics, Karolinska Institutet, Stockholm, Sweden

Historical

In 1927, Hesse[1] showed that the Flexner-Jobling rat carcinoma can be transmitted by ascitic fluid that develops during the growth of the tumor in the peritoneal cavity. He recommended the procedure as an easy and convenient means for tumor transplantation. This finding was confirmed one year later by Koch,[2] who found that this method of transmission results in an increased percentage of takes when compared to subcutaneous transplantation. Koch was trying to prove in his experiments an assumed protozoan etiology of cancer, and many of his arguments have lost interest. Some of his findings are still important, however, particularly his description of the gradually increasing cellularity of the ascitic fluid with time, the preservation of transmissibility upon cold storage, the loss of activity after exposure to 37° C. and after filtration, the technique to obtain normal peritoneal fluid from healthy rats for comparative studies; also the emphasis placed on the necessity of *wet* fixation to preserve cytological detail in the exudate cells. Two of Koch's collaborators, Loewenthal and Jahn,[3] have tried repeatedly to perform similar experiments with the Ehrlich mouse carcinoma, but were at first quite unsuccessful in their efforts. In the course of three years, they failed to obtain other than solid growth after the intraperitoneal inoculation of the tumor. In 1932, using the same procedure, they suddenly obtained a carcinomatous ascitic fluid containing a large concentration of neoplastic cells. They established a new transfer line of the Ehrlich carcinoma, carried by serial intraperitoneal passage of peritoneal exudate, and called it "ascites tumor." They have pointed out the possibility of exact inoculum dosage with this material and have found a correlation between the number of cells inoculated and survival time. They have also realized that small inocula often fail to produce ascites and give rise only to solid tumors.

The ascites tumor of Loewenthal and Jahn found wide use in experimentation in Germany soon after its description and, in more recent years, elsewhere. Lettré[4, 5] one of the first to study its various characteristics in detail, emphasized the advantages of this tumor form in chemotherapeutic experiments. Klein *et al.*[6, 7, 8] have recommended it for studies on the chemical composition of tumor cells, and Warburg and Hiepler[9] were the first to use it in metabolic experiments. Its extraordinary value in studies on chromosome number and morphology was recognized by Hauschka and Levan.[10]

Following its discovery, there was a tendency to regard the ascites tumor of Loewenthal and Jahn as a very special case or variant of transplantable tumors

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that could not be imitated with other neoplasms or at other times. It was soon shown, however, that this is not so. Warren and Gates[11] could transfer the Walker carcinoma with ascites, and they found that the fluid contains between 8 and 58 million cells per ml. A thorough study on another type of ascites tumor had to wait until 1943, when Yoshida[12, 13] found a new spontaneous or perhaps induced ascites tumor in the rat. Working on this material, Yoshida found it possible to differentiate tumor cells from other cellular elements, and he studied the cellular composition of the peritoneal fluid as a function of time after inoculation. He stated that the inoculated tumor cells overgrow rapidly the normal or inflammatory exudate cells and appear as a "nearly pure culture." A large number of experiments have been conducted on the Yoshida sarcoma by various Japanese workers.[13]

In 1947, Goldie and his collaborators started a series of experiments in the United States. These investigators have demonstrated that a number of lymphomas, leukemias, and sarcomas can be grown in the form of freely suspended cells in the peritoneal fluid.[14-17] They have studied several growth characteristics of these tumor cells in various tissue environments. Since they could obtain the growth of free cells in body fluids even outside the peritoneal cavity, they prefer the term "free-cell neoplasm" to the expression "ascites tumor."

Our own interest in ascites tumors arose in 1948, when we converted a solid line of the Ehrlich carcinoma into the ascites form[18] now used as the tetraploid Ehrlich ascites tumor. Using the Krebs 2 carcinoma in 1950, we have encountered, for the first time, the phenomenon later called gradual conversion of solid neoplasms into ascites tumors.[19] Subsequently,[20, 21] a study was carried out on 42 transplantable tumors with regard to their convertibility. These experiments will be discussed below.

Recently, reports have been accumulating on new forms of ascites tumors. Interesting types have been described by Craigie,[22, 23] Makino *et al.*,[24] Tanaka and Kanô,[25] Takeda *et al.*,[26] Isaka,[27] Bather,[28] Williams,[29] Shelton,[30] and Fekete and Griffen.[31] Quantitative methods to measure the multiplication of the tumor cells in the peritoneal fluid have been worked out by Klein and Révész,[32, 33] Patt *et al.*,[34, 35] Lucké and Berwick,[36] and Eva Klein.[37] The chromosome cytology and histocompatibility relationships of ascites tumors have been studied thoroughly by Hauschka and Levan.[10, 38-42] Transplantation by means of single cells was achieved by Ishibashi,[43] Hosokawa,[44] Hauschka,[41, 42] Lettré and Querner,[45] and E. Klein.[46] The importance of single-cell clones for population analysis was demonstrated by the experiments of Hauschka.

Before proceeding to attempt an analysis of the phenomenon of convertibility of solid tumors into ascites form, I should like to clarify one point of terminology concerning the use of the expression "ascites tumor." We agree with Goldie that the term is not very adequate, since one is not dealing with a special group of tumors but rather with a particular form of tumor growth and because growth of neoplastic cells in suspension may occur in other body cavities besides the abdominal. We do not know of any better term, however. Free-cell neoplasm does not appear to be adequate either, since there are perfectly good ascites tumors that grow in the form of cell complexes rather than free cells, such as the TA3 and MC1A mouse tumors[20] and the rat ascites hepatoma.[47] Since the terms "ascites tumor" or "ascites form of tumor growth" are widely used in the literature, we have adhered to these terms for historical reasons and, because of the lack of more adequate alternatives, we shall use them in the present discussion. We feel, however, that they should be used in the technical sense, if at all, and this makes a definition necessary. As before, we should like to restrict the term to cases analogous to the classical ascites tumor of Loewenthal and Jahn, *i.e.*, a tumor in which active multiplication of free neoplastic cells and/or cell complexes can be shown to occur in the peritoneal fluid, leading to a high absolute and relative concentration of tumor cells, that is to say, to a nearly pure culture. Tumors transmitted by peritoneal exudates should not be necessarily called ascites tumors unless they qualify under these criteria. In the following discussion, the term will be used in this sense, and the ascites form of tumor growth will be abbreviated as A-form.

Survey of Mouse Tumors

We have surveyed 42 different mouse tumors with regard to their convertibility to A-form.[21] Briefly, the following procedure was used: solid tumors of various origins (TABLE 1) and, in most cases, homologous to one of a number of inbred strains were minced mechanically, and 0.2–0.5 gm. of the fresh tumor pulp was injected intraperitoneally into mice belonging to the strain of origin or suitable F_1 hybrids. The tumor was then maintained by successive intraperitoneal transfers for at least 10, but in most cases up to 30 to 40, transfer generations. While carrying out successive transfers, inoculations of peritoneal exudate in 0.4 ml. amounts were made whenever possible. Exudate transfers were counted as FTG's (fluid transfer generations). Each exudate was examined on stained smears for tumor-cell content. If no exudates were available, solid-tumor mince was again used for transfer.

TABLE 1
TUMORS GROUPED ACCORDING TO TYPE

Tumor type	Total number	Transformation into ascites tumor		
		Successful		Unsuccessful
		Immediately	Gradually	
Spontaneous mammary adenocarcinoma	17	0	0	17
Transplanted mammary adenocarcinoma	6	0	4	2
Transplanted sarcoma originally induced	4	0	2	2
Transplanted sarcoma originally spontaneous	4	1	0	3
Transplanted lymphoma	5	5	0	0
Transplanted melanoma	2	0	0	2
Transplanted hepatoma	2	0	0	2
Transplanted neuroblastoma	1	0	0	1
Transplanted chondrosarcoma	1	0	0	1
Total	42	6	6	30

The different tumors fell into three categories in regard to convertibility. Some of them gave rise to typical ascites tumors immediately after their first intraperitoneal inoculation (FIGURE 1). Others had to be carried for a number of FTG's before suspended tumor cells appeared in any considerable number in the exudate (FIGURE 2). The number of FTG's necessary to achieve conversion to A-form varied between 4 and 24 for these neoplasms. Still other tumors—the majority—remained inconvertible to A-form, in spite of extensive trials. The behavior of the different tumors is summarized in TABLE 1, where they are grouped according to histological type, and in TABLE 2, where they are classified according to the median survival time of mouse groups receiving 0.2–0.5 gm. minced tumor intraperitoneally. Median survival time can be considered as a rough indicator of growth rate.

The data in TABLE 1 suggest that there is no simple relationship between convertibility and morphogenetic origin, if exception is made for the regularly

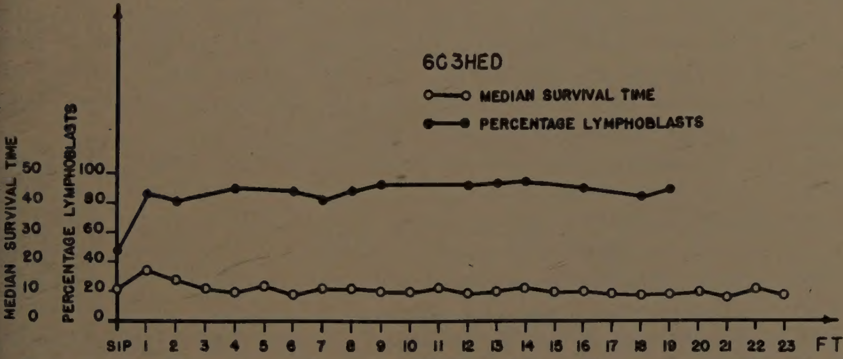

FIGURE 1. Immediate conversion of lymphoma 6C3HED into A-form. The percentage of malignant lymphoblasts was determined on stained smears around the median survival time. SIP: intraperitoneal inoculation of solid tumor. FTG: fluid transfer generation.

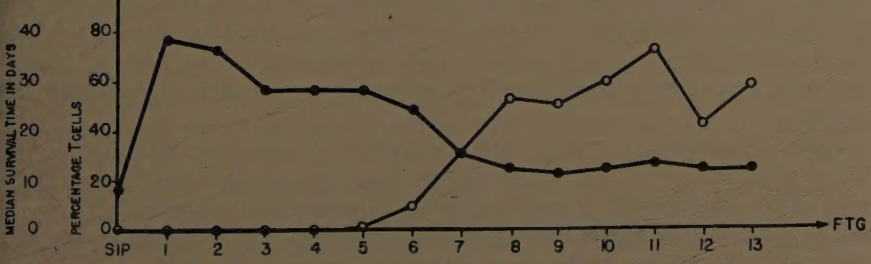

FIGURE 2. Gradual conversion of carcinoma TA3 into A-form. Symbols as in FIGURE 1.

TABLE 2

TUMORS GROUPED ACCORDING TO THE MEDIAN SURVIVAL TIMES OF INTRAPERITONEALLY INOCULATED ANIMALS

Median survival time in days	Total numberof tumors tested	Transformation into ascites tumor	
		Successful	Unsuccessful
6–10	7	5	2
11–15	6	5	1
16–25	5	2	3
26–35	6	0	6
Over 35	18	0	18
Total..................	42	12	30

convertible lymphomas. It appears from TABLE 2 that conversion occurs only with the rapidly growing tumors, but not invariably even here. The tumors belonging to the more rapidly growing categories were all highly anaplastic and had previously passed through a long series of serial passages before they were used for the present experiments. It would appear that a rather high growth rate and/or high degree of anaplasticity are necessary, but in themselves not sufficient conditions for convertibility to A-form.

We have accordingly raised the question as to whether convertibility depends on the capacity of the various tumor cells to survive and grow under the environmental conditions of the peritoneal fluid or, rather, on the ability of the host animal to respond with exudate formation to the inoculation of the different types. Suspensions prepared from nine convertible and eight inconvertible tumors were tested with regard to cell survival within pre-established ascites during a period of two days.[48] A clear correlation was found between survival and convertibility, indicating that the latter depends primarily on some variable quality of the tumor cells that permits their survival within the peritoneal fluid.

The Mechanism of Gradual Conversion

For a more detailed analysis, the case of gradual conversion appeared to be particularly interesting. Since it is a reproducible quality of certain tumor lines that occurs under standardizable conditions and is usually extended through a sufficiently prolonged period of time, it is one of the few cases of "tumor progression" amenable to experimental study at the present time. Furthermore, comparisons between the original and the transformed sublines of the same tumor, grown under similar environmental conditions, permit investigations on the morphological, biochemical, or growth correlates of the change.

Some representative cases of gradual transformation were subjected to detailed analysis.[37, 49, 50] Our primary aim was to decide between the two main alternative mechanisms of selection *versus* physiological adaptation. According to the first viewpoint, transformation is the result of a change in one or a few cells that renders them better fitted for survival, or results in a higher growth rate under the environmental conditions imposed and leads thereby to their selective concentration. The changed cells may or may not be present in the population when the experiment is started, but their appearance is "spontaneous." The role of the specific environment is purely selective and not inducive. According to the second possibility, a physiological adaptive change is induced by the invironment in the majority of the population by some such mechanism as the " . . . . expansion of the appropriate enzymes or the development of already existing metabolic routes . . . ."[51] The two theories are not always mutually exclusive, as a superimposed adaptation may be induced by the environment in variant cells selected because of their greater adaptability. The basic difference between the two alternatives lies in the fact that a selection mechanism results in the "heterogeneous or discontinuous" adaptation of the population, as only a fraction of the cells contributes to the adaptive process, while a physiological adaptation in the narrower sense means the participation of the majority ("homogeneous or continuous" adaptation).[52]

A number of experiments conducted on the mechanism of gradual transformation have provided conclusive support for the selection process.

The following were the main experimental results:

Once attained, the change is stable and irreversible, even during prolonged periods of propagation in the original solid form. This has been shown with three representative tumors of the gradually convertible group.[37,49] The ascites forms of the Krebs 2 carcinoma, MC1M sarcoma, and MC1A sarcoma, reinoculated subcutaneously, were carried in the solid form for 25, 36, and 45 transfer generations respectively. These so-called AS-lines remained immediately convertible to A-form in sharp contrast to the original unconverted solid (SS) lines that can give rise only to solid growth after their first intraperitoneal inoculation. In a number of cases, the permanence of the change has been studied quantitatively, with the help of growth curves. For the MC1M sarcoma, this is demonstrated in FIGURES 3 and 4. FIGURE 3 shows the development of the three sublines. M_{SS} is the original solid line, M_{AA} the ascites line, established by 10 generations of serial intraperitoneal exudate-transfer. Line M_{AS} has been derived from M_{AA} by subcutaneous inoculation of ascites, and is maintained by serial subcutaneous transfer. FIGURE 4 shows the total number of free tumor cells, followed as a function of time, after the intraperitoneal inoculation of one million viable cells (estimated by the eosin technique of Schrek) from the different lines. It appears that there is no difference between the growth of M_{AA}, M_{AS_1} (solid tumor of the 1st generation) and $M_{AS_{15}}$ (solid tumor of the 15th generation). In contrast, the curve of M_{SS} demonstrates that cells of the *original* solid line are not capable of multiplying in the peritoneal fluid after the inoculation of cell suspensions that have been prepared in the same way and contain the same number of viable cells. It has to be emphasized that all animals inoculated with M_{SS} died eventually with large solid tumors of the peritoneal cavity and/or the abdominal wall. Usually these animals also had large hemorrhagic exudates that were always very poor in tumor cells. The MC1M sarcoma originates in the C3H strain, and all its sublines are maintained in C3H mice or F_1 hybrids with C3H as one of the parents. All sublines grow progressively in 100 per cent in these hosts. The differences in their intraperitoneal behavior, therefore, cannot be explained on an immunogenetic basis.

The similarity of the growth curves of M_{AA} and $M_{AS_{15}}$ indicated that 15 subcutaneous passages in the solid form did not alter the ability of the ascites cells to grow in the peritoneal fluid to any measurable degree. Thus far, M_{AS} has passed through 38 subcutaneous transfers without any change in this

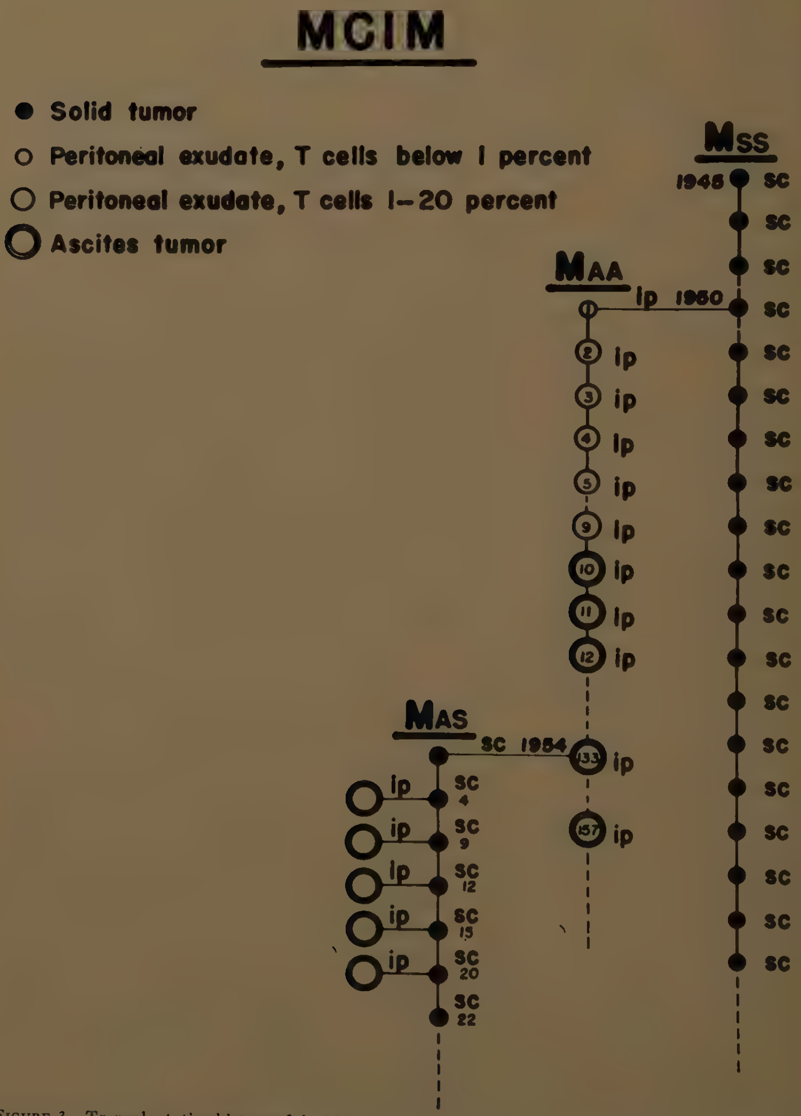

FIGURE 3. Transplantation history of the MC1M sarcoma sublines. For M_{AA} and M_{AS}, the figures denote the number of serial passages.

ability. Similar findings are available on the MC1A sarcoma after 45 subcutaneous passages in the AS form. Besides growth curves, comparisons were also made between the critical inoculum levels needed for 100 per cent primary ascites production with the AA and the serially carried AS lines respectively. No difference was found, both forms having a similar critical level of the order of 1 to 4 × 10^5 cells. Thus, all attempts to detect reverse changes towards the SS type have given negative results so far.

The permanence of the transformation reduced the probability of a purely adaptive change and stimulated investigations to test the selection hypothesis. Several different experiments were carried out. They were intended to study the effects of increasing the presumed selection pressure, to test the possible

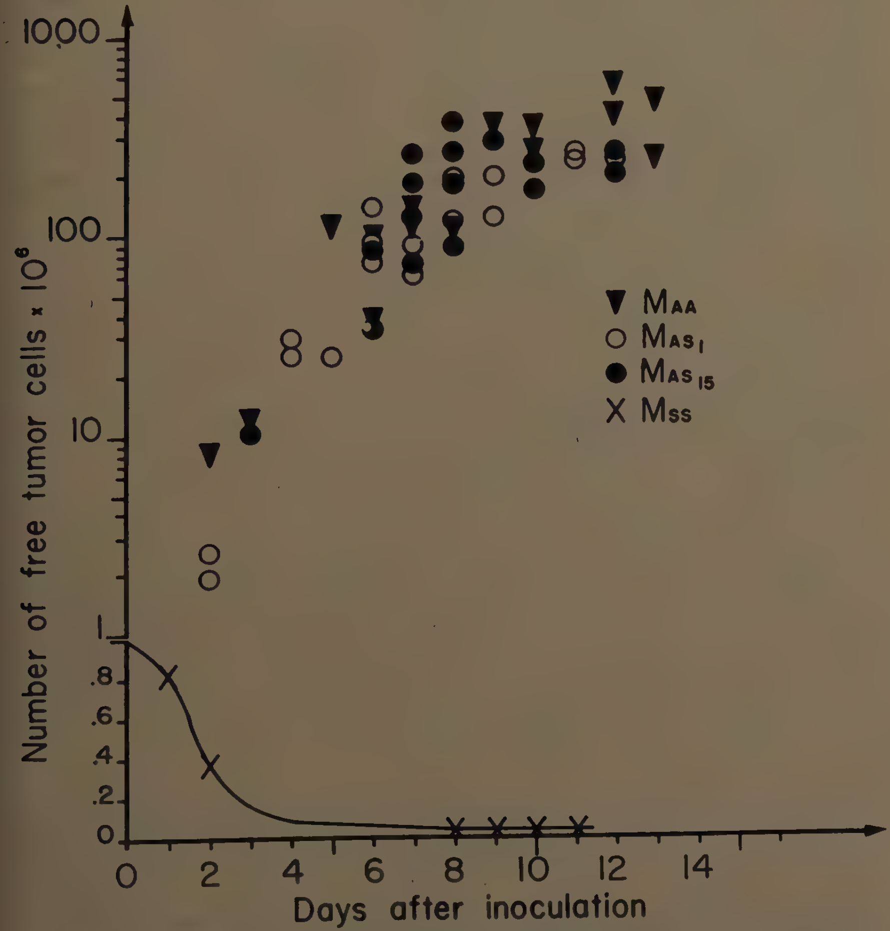

FIGURE 4. Total number of free tumor cells in the peritoneal fluid after the inoculation of one million eosin-unstained cells, derived from various sublines of MC1M sarcoma as indicated. All determinations have been made by a quantitative rinsing technique, combined with cell counting and differential counting.[32] Arithmetic scale $0\text{–}10^6$, logarithmic scale $10^6\text{–}10^9$.

heterogeneity of the original solid tumor, and to imitate the conversion process by means of artificially mixed populations. All this work was done with the MC1A and MC1M sarcomas.

The experiments with the MC1A sarcoma are summarized in FIGURE 5. The same three basic lines exist as for MC1M, showing the permanence of the ascites transformation (A_{SS}, A_{AA} and A_{AS}).

The ascites line (A_{AA}) was derived from the original solid line (A_{SS}) by 24 generations of intraperitoneal exudate transfers. During the earlier part of this process, the exudates used for inoculation were secondary to the growth of solid tumors in the peritoneal cavity and contained only few tumor cells. The dose of inoculum was thus rather low (of the probable order of 10^4 to 10^5 cells). It has been shown, however, that even for typical ascites tumors the use of large inocula is often a prerequisite to obtain a primary multiplication of free tumor cells in the peritoneal fluid of adult mice, while small inocula give rise mainly to solid tumors.[6-8, 18, 50] The "critical" inoculum level necessary for 100 per cent growth in A-form shows great variations with different tumor-host combinations. For A_{AA} in C3H mice, it is of the order of 2 to 4 × 10^5 tumor cells. Smaller inocula lead only seldom and irregularly to typical ascites tumors, and these usually can be shown to follow the ulceration of solid tumors towards the peritoneal cavity in a secondary way.

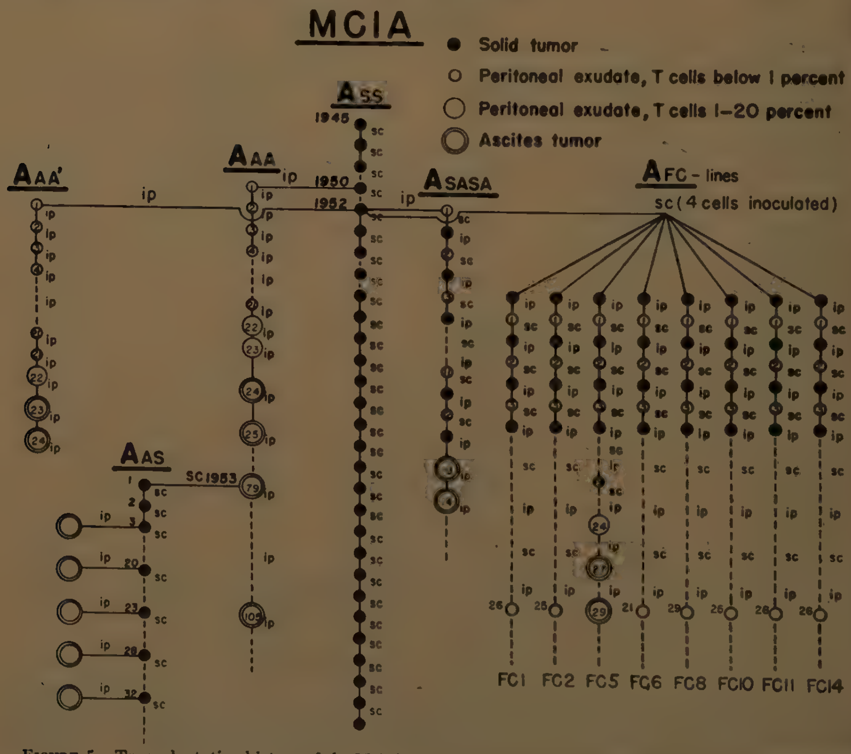

FIGURE 5. Transplantation history of the MC1A sarcoma sublines. The small figures denote the number of serial transfers.

In terms of the selection hypothesis, the selective advantage of variant tumor cells possessing an increased capacity to survive and grow in the peritoneal fluid would thus be reduced by the necessity of using small inocula during the earlier part of the serial exudate transfers. If the inoculum dose could be increased, the number and, consecutively, the selective advantage of these cells could be expected to rise and thereby reduce the number of "fluid transfer generations" necessary to achieve the ascites transformation.

These arguments served as the theoretical basis to start the tumor line denoted A_{SASA}, the transplantation history of which appears in FIGURE 5. This line originates from A_{SS} and has been carried by alternating intraperitoneal and subcutaneous transfers. In every second generation, solid tumors were inoculated intraperitoneally in large amounts (0.2 to 0.4 gm. tissue per mouse). This resulted in the development of solid abdominal tumors, followed by exudate formation. The exudate, being usually very poor in tumor cells, was then inoculated subcutaneously in the interposed transfer generations and regularly gave rise to solid tumors. Thus these solid tumors essentially served the purpose of enriching the few tumor cells in the exudate and to enlarge inoculum dose before every intraperitoneal transfer.

Simultaneously with A_{SASA}, a control line was started from the same inoculum ($A_{AA'}$) that was carried by serial intraperitoneal exudate transfers in the same way as the original A_{AA} line two years earlier (FIGURE 5).

On a selection hypothesis, the method of alternating transfers (A_{SASA}) could be expected to reduce the number of intraperitoneal passages needed to achieve ascites transformation by increasing the selective advantage of the changed cells, as discussed above. A physiological adaptation mechanism, on the other hand, probably would be counteracted by the interposed subcutaneous transfers, permitting cell growth in the original environment for one generation after each period of exposure to the environment of the peritoneal fluid. The length of the transformation period would thus be prolonged or at least unchanged.

As appears from FIGURE 5, the transformation period was actually reduced from 23 ($A_{AA'}$) to 13 (A_{SASA}) intraperitoneal transfer generations. The growth curve of the converted A_{SASA} line was closely similar to that of the established ascites line. It is of interest to point out that there was good agreement between the transformation histories of A_{AA} and $A_{AA'}$.

These findings are in disagreement with the hypothesis of a pure physiological adaptation induced by the environment, and they lend some further support to the selection hypothesis. More direct evidence could be obtained from the serial transplantation of independent A_{SS} transfer lines, started with very small inocula (A_{FC}-lines, FIGURE 5). Eight different lines were established by inoculating a statistical average of four cells into newborn C3H mice from the same pool of tumor cells that was used to start A_{SASA} and $A_{AA'}$. All A_{FC}-lines were carried by exactly the same procedure of transplantation as A_{SASA} and the size of the first inoculum was the only difference. While A_{SASA} needed 13 intraperitoneal transfers for conversion, only one out of eight A_{FC}-lines transformed to A-form after 24 intraperitoneal transfers, and seven remained inconvertible in spite of 21 to 29 similar passages. These results indicate that the majority of the cell population in the original A_{SS} solid tumor is incapable of multiplying in the peritoneal fluid. A randomly selected small inoculum therefore gives rise to tumors that are largely inconvertible to A-form, at least within the limits imposed by the number of transfers used in the present investigation.

Since the environmental conditions were identical for A_{SASA} and the A_{FC}-lines, the role of the specific environment seems to be selective rather than inducive. To explain the findings, it had to be postulated that the original A_{SS} tumor contains a relatively small proportion of variant cells possessing a discontinuously high survival value in the peritoneal fluid. Using small starting inocula would then lead to the exclusion of this fraction. The eventual transformation of one A_{FC}-line (A_{FC_5}) is most plausibly explained by assuming that mutation occurred in this line during the course of its serial transfer. It is improbable that a variant cell was already included in the original population of four cells, as it would have represented a large fraction of the population and would have been selected out very soon. In fact, the opposite was true, and transformation time was considerably prolonged for A_{FC_5} (27 generations), indicating that the change had taken place at a later stage.

A number of questions arise in connection with these results. First, it is surprising that the variant cells that are assumed to be present in the original SS-population need such a prolonged period of specific transfers (about 10 serial intraperitoneal generations in the case of MC1M, 23 to 24 with MC1A) before they can manifest themselves by growing in the peritoneal fluid. Why are they not able to grow immediately after the first intraperitoneal inoculation of the tumor? Second, if variant cells arise continuously and are apparently stable even under growth in the solid environment, why don't they gradually accumulate in the population and make the tumor immediately convertible? Third, what is the nature of the change that enables the variant cells to grow in the peritoneal fluid?

The first problem was investigated for the case of the MC1M tumor by a series of model experiments, inoculating artificial mixtures of the original solid line (M_{SS}) and the established ascites line (M_{AA}) in known proportions, and following them through the usual procedure of serial intraperitoneal exudate-transfers. The results are given in TABLE 3. It appears that when the proportion of M_{AA} cells was 5 per cent or less, growth in A-form could not be obtained immediately, but only after a number of transfers. A high correlation exists between this number and the logarithmic proportion of M_{AA} cells in the inoculum. Thus, the gradual transformation process can be imitated with known mixtures of the converted and the original lines. This proves one postulate of the selection theory, namely, that a small proportion of variant cells possessing discontinuously high survival and growth capacities in the peritoneal fluid do indeed remain masked when the tumor is inoculated into the peritoneal cavity and that a certain number of selective exudate-transfers is necessary before they can become manifest by changing the characteristics of the population.

Since about 10 exudate-transfers are usually needed to convert M_{SS} into M_{AA}, the approximately rectilinear relationship between the logarithmic values of column 3 and the figures in column 4 (TABLE 1) was tentatively extrapolated to 10. Provided that the variant cells in the original M_{SS} population are identical with M_{AA} cells and no superimposed adaptation or other sequential

TABLE 3

NUMBER OF SERIAL INTRAPERITONEAL EXUDATE-TRANSFER GENERATIONS NECESSARY TO CONVERT KNOWN MIXTURES OF M_{SS} AND M_{AA} CELLS INTO A-FORM.

Number of M_{SS} cells per inoculum 1	Number of M_{AA} cells admixed 2	Proportion of M_{AA} cells 3	Number of fluid transfer generations necessary for conversion 4
5×10^5	5×10^5	5×10^{-1}	1
3×10^5	3×10^5	5×10^{-1}	1
6×10^5	3×10^5	3.3×10^{-1}	1
2×10^6	5×10^5	2×10^{-1}	1
4×10^5	1×10^5	2×10^{-1}	2
2×10^6	1×10^5	5×10^{-2}	2
2×10^5	1×10^4	5×10^{-2}	4
2×10^5	1×10^3	5×10^{-3}	4
5×10^5	2×10^3	4×10^{-3}	3
1×10^6	4×10^3	4×10^{-3}	3
5×10^5	2×10^2	4×10^{-4}	6
5×10^5	2×10^1	4×10^{-5}	6
$20–40 \times 10^6$			10

Calculations of regression and correlation between the data of column 4 (x) and the logarithm of the figures in column 3 (y), with exception of the last horizontal line:

$$n = 12 \quad r = -0.925$$

$$z \pm \sigma_z = -1.62 \pm 0.33 \quad P<0.001$$

$$b \pm \sigma_b = -0.660 \pm 0.085$$

$$y = -0.66\,x + 0.21$$

changes are operative during conversion, the extrapolation would indicate that the frequency of variant cells is about 1 in 2.4×10^6 in the original M_{SS} population. The experiments are being repeated on a larger scale to provide a more accurate estimate. Although the present value must be considered as tentative, it is by no means unrealistic, since an inoculum of at least 2 to 4×10^7 cells is usually employed for starting conversion lines. The findings are thus compatible with a purely selective mechanism, and a superimposed adaptation or other sequential changes do not necessarily have to be assumed to explain the results, although their existence is by no means excluded.

To explain the latency of the changed cells through several transfers, a consideration of the "critical" inoculum size appears relevant. As mentioned above, certain established ascites tumors do not grow in A-form after intraperitoneal inoculation unless the number of implanted cells exceeds a certain value. This value is different for different tumors and, in at least one case, was shown to change with time in the decreasing direction.[50] For the MC1M and MC1A ascites lines (M_{AA} and A_{AA} respectively) it is of the order of 1 to 4×10^5 tumor cells. The exact value depends on the genotype of the host, usually either a C3H mouse or an F_1 hybrid with C3H as one of its parents. Certain F_1 combinations give a higher critical value than others.[50] Other factors that may produce slight variations in the critical dose concern the general condition of the inoculated cells, primarily their physiological age and viability.

If ascites cells are inoculated intraperitoneally in subcritical doses, they cannot maintain themselves in the peritoneal fluid and disappear within a few days. Unless the inoculum is very small, the mouse will still die with one or several solid tumors that usually develop at the site of inoculation in the abdominal wall. Occasionally, these tumors may infiltrate the peritoneal cavity after having reached considerable size and may cause a secondary ascites formation. If they are inoculated intraperitoneally in large doses, ascites tumors always develop, showing the reversible and dose-dependent nature of the phenomenon.

If a high number of variant cells has to be present in the inoculum to make growth in A-form possible, it can easily be seen why a small proportion cannot become manifest in the early phases of the reconstruction experiments. Since they do become selected after a number of exudate-transfers, they must possess some kind of selective advantage in the peritoneal fluid, in spite of the fact that they cannot grow into a concentrated culture. The exudate that is used for these passages develops secondarily to the solid tumorous growth in the cavity and contains a low number of tumor cells (of the probable order of 10^4 to 10^6 per ml.). These presumably must have derived by desquamation from the surface of infiltrating tumors, since they cannot be detected before considerable infiltration is present. Such a sample of desquamated tumor cells may be a random representative of the population, but it is also possible that the desquamation of the variant cells is favored by their lower degree of adhesiveness.[37] If this is not the case, their proportion might still gradually increase in the exudate samples used for transfer, provided that they survive for a longer period in the suspended form than the original cells and possibly undergo a few divisions. Other experiments[48] have shown conclusively that differences may exist in the survival capacities of different kinds of tumor cells within pre-established peritoneal exudates.

In the course of the gradual conversion process, there is little increase in the average concentration of free tumor cells during the early transfer generations when the proportion of variant cells is so low that their absolute number remains subcritical in the inocula used for transfer. When the critical proportion is once attained, the variant cell type suddenly becomes able to *grow* in the fluid, its selective advantage increases abruptly, and conversion follows within a few generations.

Another problem concerns the fact that the solid SS-lines of both MC-sarcomas remained gradually convertible to A-form during an observation period of four years and that no major change could be observed in the number of serial exudate-transfers required for conversion. Apparently, the variant cells do not accumulate with time in the solid tumors to any detectable degree, indicating the existence either of reverse changes or of adverse selection. The experiments with the serial AS-lines have so far failed to supply evidence for the existence of reverse changes. Since both the original and the converted cells have the same growth rate in the solid form,[48] it is not necessary to assume the existence of any selective pressure when they grow together. Since one type might still possess competitive advantages over the other in the mixed culture, an experiment was carried out involving the serial *subcutaneous* transfer of a 1:1 mixture of M_{SS} and M_{AA} cells. On each generation, the capacity for growth in A-form was tested by the intraperitoneal inoculation of 2 to 4 × 10^7

cells. Until the sixth generation, typical ascites tumors developed regularly. After the seventh solid passage, only three out of five mice had ascites tumors, and two died with solid neoplasms. From the eighth generation, no ascites tumors could be obtained at all, and intraperitoneal inoculation gave rise exclusively to the development of solid tumors and hemorrhagic exudates, the latter containing a low concentration of predominantly inflammatory cells. This picture is similar to that obtained after the intraperitoneal inoculation of M_{SS} alone.

This experiment suggests that the transformed M_{AA} cells may be at selective disadvantage when growing together with M_{SS} cells in the solid form. In the course of seven or eight transfers, the proportion of M_{AA} cells must have decreased so as to render their number subcritical in the test-inoculum. With the inoculum size used, this means that the original proportion (5×10^{-1}) of M_{AA} cells has fallen to about 10^{-2} after seven or eight subcutaneous passages.

This finding explains why the solid SS-lines do not become immediately convertible in the course of time. It might be questioned, on the other hand, why the solid lines derived from the M_{AA} and A_{AA} ascites tumors (AS-lines) did not revert to the SS type in spite of 36 and 45 generations, respectively, of subcutaneous transfer. The probable explanation is given by the quantitative differences between the two types of experiments: the presently investigated mixed population contained 50 per cent M_{SS} cells in the beginning, while the M_{AS} and A_{AS} populations start out as presumably pure cultures of AA-type cells. The frequency of reverse changes, if they occur at all, might be very low and, since the selective pressure does not appear to be very high, a considerable period of time may elapse before possible reverse changes can become manifest in the AS transfer lines.

The question arises as to the nature of the change that renders certain tumor cells capable of growth in the peritoneal fluid. Since the change is stable and hereditary, and occurs apparently spontaneously and probably at a low rate, it was tentatively called mutational, using the term in its broadest sense and without regard to its intrachromosomal or extrachromosomal nature. Studies conducted in order to find its possible morphological or biochemical correlates (using mainly the directly comparable solid M_{SS}-M_{AS} or A_{SS}-A_{AS} lines as test systems) have led to the following results: conversion to A-form appeared to be correlated with an increased metastasizing tendency to the lungs,[37] a reduced cellular adhesiveness,[37] an increased mobility in tissue culture,[53] certain cytochemical changes in the microsome fraction,[50] and an improved capacity to survive in a "starvation" medium *in vitro*.[50] There was no change in average nuclear size and general histological picture, with the exception of an increase in the proportion of round as contrasted to spindle-shaped cells.[49] In the MC1A rhabdomyosarcoma, there was no change in calcium or myosin content.[37]

The nutrition of ascites tumor cells suspended in the peritoneal fluid presents an interesting problem. Nutrients are supplied by the vessels under the peritoneal surface and are carried probably by diffusion through the double barrier of the capillary wall and the peritoneal surface. Intestinal peristalsis and the movements of the mouse are continuously mixing the peritoneal fluid and, within it, no diffusion gradients can be assumed to exist. An injected dye was shown to become homogeneously distributed through the fluid within three to five minutes.[33] Thus, all suspended tumor cells have to share equally the available pool of nutrients in any given moment. They are also exposed equally to the breakdown products of all other cells. One may postulate that with the usual increase of cell number to 10^8 to 10^9 there must be a great shortage of nutrients available per cell and per unit time in the fully developed ascites tumors. There are some experimental findings available to support this contention. Warburg and Hiepler[9] have shown that the supernatant of the Ehrlich ascites tumor contains very little sugar and that anaerobic conditions prevail within the fluid. According to these authors, the oxygen and sugar consumption of the tumor cells is not regulated by their metabolic capacity, but by the limiting amounts present in the fluid. They find it surprising that Ehrlich ascites tumor cells can survive in the peritoneal fluid at all, and they raise the question as to what amounts of energy are sufficient to keep the cells surviving (and, we may add, multiplying) *in vivo*. We have been able to confirm the findings of Warburg and Hiepler regarding glucose content. If glucose concentration is followed in the peritoneal fluid as a function of time after the inoculation of different cell numbers and correlated with the growth curve (FIGURE 6), it is seen to fall rapidly and reach a very low level already at a total tumor-cell number of 10^7. Similar relationships may possibly exist for other important nutrients. If cell multiplication would stop at the level of 10^7, the

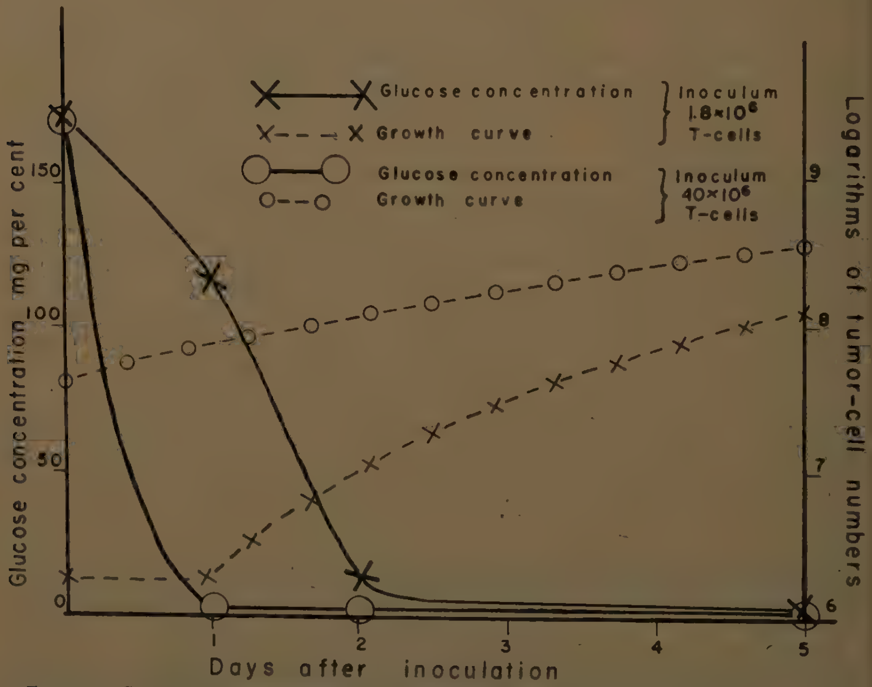

FIGURE 6. Concentration of glucose and total number of free tumor cells in the peritoneal fluid as a function of time after the inoculation of 1.8 × 10^6 and 40 × 10^6 Ehrlich ascites tumor cells, respectively.

tumor would not classify under the definition of an ascites tumor. If a survey is made of available studies on the growth curves of ascites tumors, it appears that cell multiplication is definitely slowing down at a total cell number of about 10^8 in Ehrlich and MC1M,[32] 2×10^8 in Krebs 2,[35] 7 to 9×10^8 in three lymphomas,[33] and comes to a standstill soon thereafter at a level of 5×10^8 in MC1M and 1 to 2×10^9 in the lymphomas while it continues at a gradually decreasing rate until the death of the host in Ehrlich and Krebs 2, reaching a final maximum value of 10^9 to 2×10^9. It appears reasonable to suggest that the average ascites tumor cell lives under great nutritional shortage, at least during the later period of the growth cycle, after the total amount of fluid has reached 3 to 4 ml.; *i.e.* a total cell number of 4 to 8×10^8 in the carcinomas and sarcomas, and 9 to 12×10^8 in the lymphomas. It is usually at or after this stage that the cells are being used for routine transfer.

Considering now the nutritional conditions under which the cells responsible for the continuous maintenance of solid tumors are living, it is obvious that diffusion gradients will make these greatly inhomogeneous. At least those cells that are lying in the immediate neighborhood of nutrient vessels can be assumed to be under more favorable nutritional conditions than the average ascites cell, however, since they receive their nourishment directly from the vessel and can take their share immediately and more according to their metabolic requirements. The same argument could be modified to apply to the wash-away of toxic breakdown products. When the solid tumor is then transplanted, these optimally situated cells will have the largest chance to survive and become represented in the stem line of the tumor. Schematically, one could say that the stem-line cell of the ascites tumor is living under the average conditions of the population (nutrients available per unit time being divided equally per cell), while the stem-line cells of the solid tumor are living under optimal conditions (nutrients available per unit time being first used by the privileged class of suitably located individuals).

These considerations have led to the speculative assumption that the mutational change leading to the ascites transformation may be essentially metabolic, involving the development of a more flexible and economic type of metabolism and/or less exacting nutritional requirements. Experiments are in progress to approach this problem, and preliminary results of *in vitro* incubating M_{AS} and M_{SS} suspensions in a starvation medium have actually shown a difference in their survival capacities, in favor of the former.[50]

As discussed above, the transformed AA-type cells are under selective disadvantage if grown together with the original SS-type in the solid form. This apparently contradicts the nutritional hypothesis. From studies on the biochemical genetics of microorganisms, a number of cases is known, however, showing that "... . mutations producing apparent increases in biochemical activities may prove unfavorable for survival under ordinary circumstances, although useful in the exceptional environment."[54] A mutant may possess selective advantage in certain environments that are deficient in metabolites or contain substances toxic to the original type. It may also maintain itself with great stability when grown alone in the original environment, but may still be overgrown by the original type when mixed and grown together under normal conditions of culture (cp., *e.g.*, Berger and Wyss[55]). It could be speculated that the competitive advantage of the original SS-type cells over the AA-type depends on a more economic type of metabolism that takes advantage of the metabolites supplied by the host while the mutant synthesizes one or several substances present in the original but not in the exceptional environment. As shown by Lwoff[56] and, in the case of tumor cells, emphasized by Hauschka,[41] a "parasitic type of metabolism involving the absence of certain syntheses may have selective advantages . . . . in a medium such as the host environment which contains enough ready-made metabolites. The advantage of not performing certain syntheses may lead to an acceleration of some other essential growth reactions."[41]

Immediate Conversion

The tumors showing immediate conversion can be subdivided into two main categories. One comprises all lymphomas and leukemias so far tested[57, 14-17, 20-21, 30] and some spontaneous or induced rat sarcomas.[12, 24, 26, 58] These tumors could readily grow in A-form irrespective of their transplantation histories and sometimes even directly from the primary tumor.[12, 58] It has to be assumed that the cell populations of these neoplasms, or at least large fractions thereof, are able to grow in the peritoneal fluid without any particular adaptation or mutation. The second category includes the immediately convertible mouse carcinomas and sarcomas so far described (Ehrlich, sarcoma 37, sarcoma 180, epithelioma 2146, *etc.*). These tumors all had long transplantation histories before their conversion into ascites form was attempted. At least two cases are known where the capacity for immediate convertibility developed suddenly in a previously inconvertible tumor during prolonged serial transfer in the solid form. These are the Ehrlich carcinoma of Loewenthal and Jahn[3] and the S3A carcinoma, followed by one of us[49] from its spontaneous origin in the inbred A strain through more than 80 passages in the home strain. The latter neoplasm was entirely inconvertible to ascites form during its first 27 transfer generations. In one of four transfer lines a change occurred somewhere between the 27th and the 60th generation that rendered immediate conversion possible. No support was obtained for the continued existence of the original inconvertible cells in the population by the 64th transfer generation in a preliminary experiment which resulted in the isolation of three single-cell clones from the solid tumor line. The change was not correlated with any alteration of growth rate and degree of differentiation. There was a certain decrease of genetic specificity. Contamination with another ascites tumor could be critically excluded. Although the mechanism of the change remains obscure, it might be speculatively attributed to the appearance of a new cell type, possessing competitive advantage over the original population if grown in the solid form and, in addition, having the capacity to grow in the ascites form when inoculated into the peritoneal cavity. This would be different from the case of gradual conversion, where the frequency of the changed cells remains low in the solid tumor, presumably because of their competitive disadvantage under such conditions and where conversion can be achieved only under deliberate selective pressure. While the mutation-selection mechanism was well documented for the case of gradual conversion, it remains purely speculative for the present case, and other interpretations cannot be excluded.

Inconvertibility

The term "inconvertibility" is somewhat arbitrary. It means only that the tumors in question have not acquired the capacity for immediate conversion so far and that gradual transformation could not be achieved by techniques that were successful with other neoplasms. Nothing is said about the future of these tumors. In fact, the S3A carcinoma was inconvertible during its early transfer history, as was mentioned, and so was the original Ehrlich tumor of Loewenthal and Jahn. All that can be said is that certain tumors appear inconvertible during the stage in their transplantation history when they are tested. Some regularities appear in this group. All highly differentiated and slowly growing mouse carcinomas and sarcomas tested with short transplanation histories belong to this group.[21] It can be postulated that the mutational change rendering growth in A-form possible does not occur or has not occurred yet in these tumors, or that it occurs at such a low frequency that the mutants are lost during transplantation. It is of interest that a few long transplanted tumors such as the C 1300 neuroblastoma and the C 954 hepatoma also belong to this group. Both tumors originated in 1940, and C 1300 is rapidly growing and anaplastic. One is tempted to the assumption that on the particular genetic background of these tumor cells the mutational change required for convertibility either does not occur or cannot reach full expression.

Conclusions

Trying to summarize the findings and visualize the interpretations, we have composed a scheme that is undoubtedly rough and oversimplified (FIGURE 7). It serves only to delineate some main groups that can be clearly recognized at the present time, and it is very probable that the picture will become considerably more complicated as new evidence accumulates. It is clear, however, that transplantable mouse and rat tumors can be classified as immediately convertible (group I); gradually convertible (group II); and inconvertible (group III). All these expressions apply to the specific time in their transplantation history when the tumors are tested, and it is not inferred that they do not change before or after this particular point of time. Actually, the immediately convertible group can be subdivided into two subgroups: those that are immediately convertible already at the primary stage (Ia), such as lymphomas and some rat sarcomas, and those that develop the capacity for immediate conversion during prolonged serial passage in the solid form (Ib) as, for instance, the S3A and probably also the Ehrlich carcinoma. Talking now in terms of cellular behavior, three main types are considered in the scheme. One type is unable to grow in the peritoneal fluid, irrespective of inoculum dose. As we have shown previously,[48] this group could again be subdivided into cells that cannot grow but survive for a period of at least two days in pre-established exudates, and cells that cannot even survive under these conditions. To simplify the presentation, no consideration has been given to this subdivision in the scheme in FIGURE 7, since nothing is known about the proportion of these

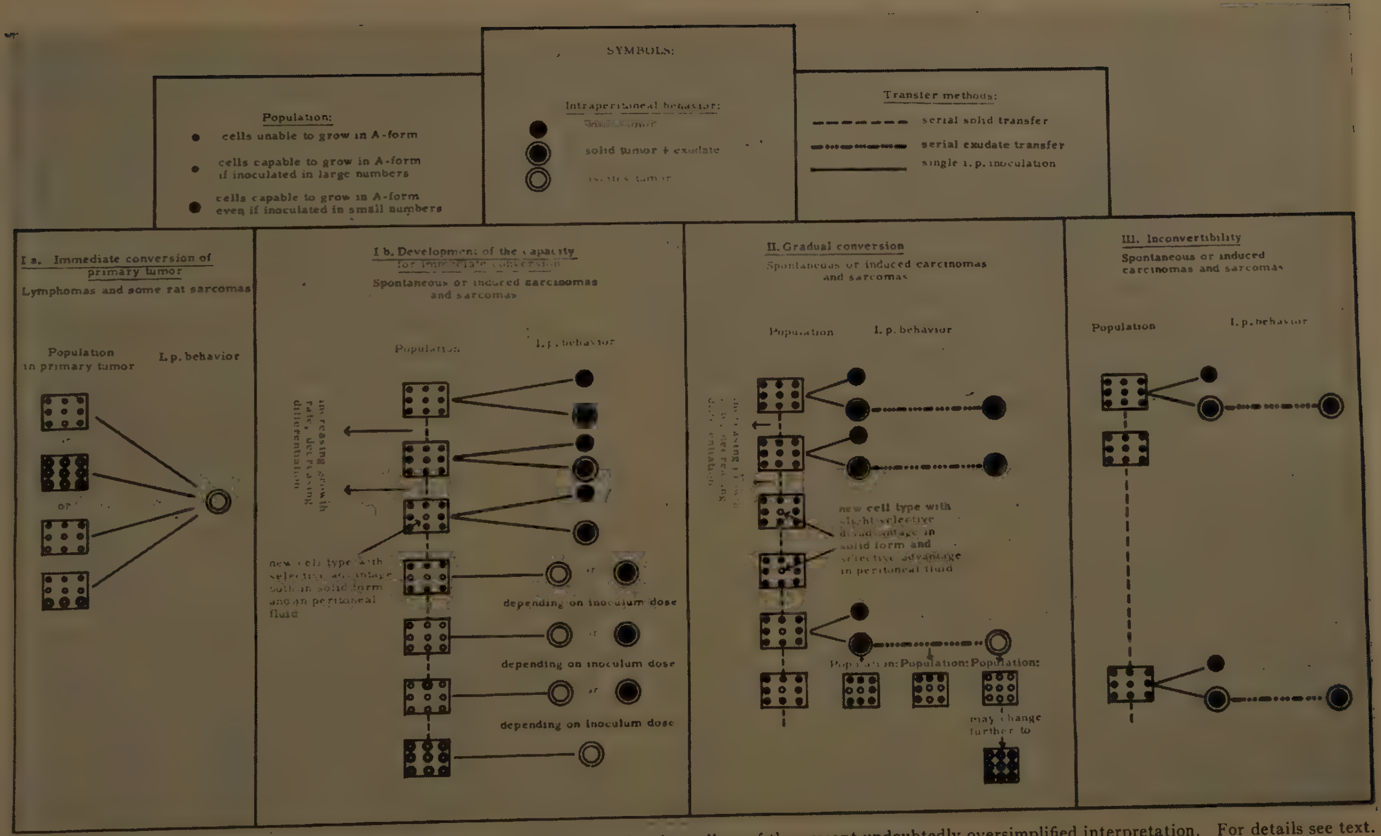
Population:
cells unable to grow in A-form
cells capable to grow in A-form if inoculated in large numbers
cells capable to grow in A-form even if inoculated in small numbers
SYMBOLS:
Intraperitoneal behavior:
solid tumor
solid tumor + exudate
ascites tumor
Transfer methods:
serial solid transfer
serial exudate transfer
single i.p. inoculation
I a. Immediate conversion of primary tumor
Lymphomas and some rat sarcomas
Population in primary tumor
I.p. behavior
or
I b. Development of the capacity for immediate conversion
Spontaneous or induced carcinomas and sarcomas
Population
I.p. behavior
increasing growth rate, decreasing differentiation
new cell type with selective advantage both in solid form and in peritoneal fluid
or
depending on inoculum dose
depending on inoculum dose
depending on inoculum dose
II. Gradual conversion
Spontaneous or induced carcinomas and sarcomas
Population
I.p. behavior
increasing growth rate, decreasing differentiation
new cell type with slight selective disadvantage in solid form and selective advantage in peritoneal fluid
Population: Population: Population:
may change further to
III. Inconvertibility
Spontaneous or induced carcinomas and sarcomas
Population
I.p. behavior

FIGURE 7. Schematic classification of tumors with regard to convertibility and main outlines of the present undoubtedly oversimplified interpretation. For details see text.

two types and possible intermediates in the various tumors. It might be mentioned, however, that at least in the cases so far tested, solid tumor populations of type III seem to contain mostly cells unable to survive under the conditions in question, while type II tumors contain predominantly cells that can survive but not grow. The two other cell types depicted in FIGURE 7 are those that can grow in the fluid provided that they are inoculated in a large dose (of the order of 10^5 to 10^6), and those that can grow even if inoculated in very small numbers, near or at the single-cell level. The demarcation of just these two types is admittedly arbitrary, and we should only like to visualize the finding that tumors with a clear critical cell dose can change in the course of time, and that small cell doses that lead at first only to solid tumor development may become able to produce primary ascites tumors at a later period in the transplantation history. Immunogenetic compatibility is a prerequisite in all cases depicted in FIGURE 7.

Talking in these terms, we visualize tumors of group Ia as populations where the majority or perhaps all of the cells can grow in the fluid. In some cases, a critical dose level does exist, in others it does not. Tumors of type Ib consist predominantly of cells unable to grow in the fluid. During serial transplantation in the solid form, variants appear possessing selective advantage in the solid form and also possessing the capacity to grow in the peritoneal fluid. They gradually replace the original population and render the tumor immediately convertible. In this case, no artifically employed environmental selective pressure is necessary. Later on, the tumor may change further and the critical dose level may decrease, indicating a further change in the population and its replacement by cells still better adapted to growth under the environmental conditions in the fluid. Tumors of group II start out as do those of group Ib, but differ in one respect: the variants that are better suited for life in the peritoneal fluid have, in this case, a slight selective disadvantage in the solid form and are therefore maintained at a low level. They cannot be concentrated, unless selective pressure is employed, by exposing the randomly exfoliating cells of the intraperitoneal solid tumor to the environmental conditions of the peritoneal fluid. In this way, the population gradually changes in favor of the variant cell type, and transformation is achieved when the latter reaches critical level in the inoculum. Once established, the ascites line may adjust itself further to the A-form of growth under prolonged propagation, as indicated by a decrease of the critical inoculum level. Finally, tumors of group III consist of cells unable to grow in the fluid. Variants do not arise, or rather have not arisen until the time of testing. Alternatively, there may be a strong selection pressure operating against them, and/or they may be lost during successive transfer.

Concluding, we should like to emphasize that the real picture may be considerably more complex. All kinds and degrees of superimposed physiological adaptation, multiple mutational adjustments either by point mutations or by gross changes in chromosome number and morphology, cytoplasmic mutations, *etc.*, might be operating. As suggested to us by Doctor Levan, we may be dealing with a succession of mutations regarding one or several quantitative characteristics. Concentrating on the problem of gradual conversion, we have tried to study, as a first approach, the question whether this change is modificative and adaptive in nature or whether we are selecting deviating cell types on the background of a real genetic variability. In other words, do all cells in a gradually convertible solid tumor have the same chance to adapt to the given environmental conditions and emerge in the final ascites tumor, or is this chance heterogeneously distributed with certain variant cells more favored than others? Is the role of the environment inducive or selective? We believe that we have answered this question in favor of the selection alternative, at least for the case of gradual conversion, although we have not excluded the possibility that other superimposed mechanisms may be operative. This finding has added additional emphasis to the mosaic concept of tumor-cell populations, already well established by the studies of Hauschka and Levan[38-42] on chromosome number, morphology, and immunogenetic specificity and by the work of Law[59] on drug resistance in leukemias. It would appear that mammalian tumors are not what they were often considered to be: clearly delineated clones of one cell type, but rather complex populations in which a broad range of variation permits the continuous operation of selective forces. This range favors the reproduction of the type that is best fit for the particular environmental conditions, leading to continuous evolutionary changes and culminating in such phenomena as extreme anaplasia, high growth rate, broad range of transplantability, independence from host-imposed growth-regulating mechanisms, resistance to growth-inhibiting drugs or to radiation, and development of the capacity to grow in a wide range of tissue environments and body fluids.

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PROSPECTS FOR A GENETICS OF SOMATIC AND TUMOR CELLS*

(Discussion of Doctor G. Klein's Paper)

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Since I have had no experience at all with ascites tumors, Doctor Theodore S. Hauschka's suggestion that I discuss Doctor Klein's paper had to be construed as an invitation to review the potential impact of microbial genetics on tumor studies. In fact, most of the hypothetical notions that will be summarized are the result of informal discussions with Doctor Hauschka, Doctor Klein, and others. The analogy between the ascites tumor cell and an infectious microorganism hardly needs to be stressed, and something may be gained by pursuing the analogy both in method and in concept.

Bacteria have been the subject of investigation of several types of genetic change and exchange:

Mutation and selection. Klein's paper illustrates one of the most disputed problems of bacterial genetics, the origin of adaptive mutations, usually involving resistance to drugs but also including nutritional or other biochemical modifications. By ever more precise methods, it has become possible to prove that such adaptive mutations occur "spontaneously," that is, independently of the environmental stress to which they confer improved fitness.[1, 2] By indirect selection,[3] for example, drug-resistant bacteria can be isolated without exposing them to the drug. It would be possible, if tedious, to extend this technique to tumor cells, but I doubt whether such an extension beyond these already impressive demonstrations[4] would be worth the effort that might be expended on more informative experiments.

Once given reliable procedures for the detection and estimation of mutations, we may then anticipate a more systematic application of mutagenic agents, both radiations and chemicals: at first, to facilitate the isolation of mutants that might be useful as genetic markers and, later, for the elucidation of radiobiological effects on tumor cells—the role of genetic damage in radiation injury[5, 6] and the mechanism of radioresistance, be it polyploidy[6] or an adaptive mutation itself.[7]

The role of selection in the determination of cell populations is obvious in some of Klein's and Hauschka's studies.[8] Bacteriological studies[9] have also drawn attention to the sampling effect of adaptive mutations: their origin, usually from a single cell, means that genotypes in mixed populations can become randomly fixed as effectively as by the purposeful technique of single cell isolation.

The impact of selective forces should not be deduced solely on hypothetical grounds, as many examples of anomalous selection in mixed cultures are not readily predictable from the behavior of the isolated components.[10]

Transduction.[11] The availability of a variety of genetic markers has led to

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a more thorough analysis of the famous "pneumococcus transformation" of Griffith, which has been followed by a series of examples of genetic exchange best understood as genetic transduction, namely, that fragments of chromosomes (or exogenotes) may be transferred from one cell to another. The remarkable feature of transduction is the ability of the fragment not only to survive its transportation but to replace homologous genes of the intact recipient chromosome by some mode of crossing-over. Two modes of transduction are now recognized: in the pneumococcus, the exogenotes consist of a suspension of chemically extracted DNA; in enteric bacteria, the exogenote is carried by a bacteriophage particle. The fragments are so small that only exceptionally are two markers so closely linked as to be transduced together.

In principle, either intact chromosomes or intact nuclei must also be considered as alternative units of exchange in transduction experiments, especially, since nuclei have been successfully transplanted by artificial means.[12]

The most explicit claim of transduction outside the bacteria has been that the tumorigenic quality itself may be transduced by DNA preparations.[13] Subsequently, however, Klein[14] placed suitable immunogenetic markers on the donor and recipient cells, and showed complete linkage of these with the neoplasia. Whatever this case may prove to be, it is therefore not a simple transduction. That is, the unit of exchange is more than a fragment. It remains to be verified whether it is less than a cell. A combination of physical, enzymatic (DNAase) and genetic studies should help to clarify this issue. Klein's example should encourage the intelligent use of genetic markers in other situations where one must exclude the persistence of intact cells.

These examples should stimulate many efforts at achieving transduction in tumor systems, a result that would add a powerful technique for genetic analysis. As suitable materials become available, both DNA and viruses of limited cytopathogenicity should be tried as possible vectors of exogenotic fragments.

Sexual recombination. While the sexual act of nuclear fusion has become surrounded with elaborate secondary paraphernalia in the course of evolution, the modes of its occurrence in microorganisms[15, 16] justify speculations as to the potentiality for sexual cycles in "somatic" cells. Scattered observations can be adduced for support. The decision will rest on properly designed experiments. Fusion of cells, however, has been indicated in liver cells.[17] The same authors have also described the confluence of nuclei or of metaphase spindles. Segregation is more problematical. Somatic segregation and crossing-over has been extensively studied in fruit flies[18] and in plant and microbial materials. Mosaic patches have been described from time to time in heterozygous birds and mammals,[19] but it has not yet been possible to ascribe these to chromosome or gene loss, or to a more regular process of segregation. It is hopeful, however, that somatic variegation (segregation?) is accelerated by radiations in birds[20] as well as in fruit flies[21] and microorganisms.[22, 23] Whatever the mechanism of these variegations, they still allow definite hope of diagnosing recessive genes in heterozygous organisms, if the variegation can be experimentally controlled. Since the unit processes are rare, we may have to rely on selective genetic methods; *a priori*, the hope of success is hardly less than it was for bacteria.[11, 15]

Both transduction and sex should be visualized as aspects of genetic recombination. The most suitable markers may be those that can be independently studied by breeding tests in the intact mouse, outstandingly the histocompatibility factors. Other markers currently available are only drug resistance and the ascites adaptation. We may already have some hint of recombination in studies of the effect of F-1 hybrid hosts on the histocompatibility patterns of transplanted tumors,[8, 24] if this does prove to be more than selection of spontaneous variants. The hybrid host is unique in being compatible with, but genetically distinct from, the tumors. If this effect is recombinational, it may help to use lines that are genetically better defined and, perhaps, to use additional markers such as drug resistance.

All recombination studies with tumor cells suffer from the burden of working with diploid, even polyploid material, which of course complicates the detection of recessive markers. A worthwhile preliminary to these studies would be the surveillance of *heterozygous* tumors, preferably derived from and maintained in F-1 hybrids of Snell's well-defined isogenic-resistant lines, to verify the occurrence and control of possible segregation, as might be revealed by occasional takes in the parental lines.

It would be difficult to estimate the importance of the accomplishment, the possibility of exact genetic analysis of tumors and tissues. Nothing less is likely to resolve the genetic bases of differentiation and of neoplasia.

Virus infection.[11, 25] Studies with lysogenic bacteria and bacteriophages have lately verified an old speculation, familiar to students of cancer viruses, that virus infection is also a species of genetic recombination. First of all, at least one virus, lambda, in *Escherichia coli* K-12 is associated with a specific nuclear site: crosses of lysogenic with sensitive bacteria show that this property segregates in the same fashion as any other mutation, being linked to a locus for galactose fermentation. But, without further evidence, it should not be assumed that other symbiotic viruses do or do not have a locus in the nucleus. Second, the mere presence of a symbiotic virus sometimes confers a unique property on the bacterium that carries it. The new property may relate directly to virus infection or it may concern the formation of exotoxin in diphtheria, or the somatic antigen in group E *Salmonella*.

Cytoplasmic factors.[26, 27, 28] Extranuclear genetic factors (plasmids) have not yet been definitely ascertained in bacteria, but are important features of the genetics of other microorganisms. So far, it has not been possible, for example, to reintroduce chloroplasts into albino green plant cells, or respiratory granules into cytochrome-oxidase-deficient yeast, by exogenous "infection." Whether mitochondria have genetic functions has been debated. For this reason, Lettré's account[29] of the reconstitution of ascites tumor cells that have been deprived of their mitochondria is of extraordinary genetic interest, as it would open the possibility of studying heterologous combinations of mitochondria and cells, a new type of genetics. Regrettably, this work has not yet been subjected to independent confirmation. When it is, genetic markers should be used to exclude the persistence of intact viable cells that otherwise escape notice, as well as to explore genetic diversification of the cytoplasmic granules.

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GROWTH OF CELL SUSPENSIONS IN TISSUE CULTURE

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In earlier publications,[1, 2] preliminary methods and concepts were described by which it had been possible to demonstrate the proliferation of strain L-929 cells as a free-floating cell suspension maintained in a rapidly agitated fluid culture medium.

In these exploratory studies several factors were given special attention. These were:

(1) It was obviously necessary to keep the cells in suspension in the nutrient fluid. To accomplish this, rapid agitation of the nutrient fluid was used. To assist in this, viscosity of the culture fluid was slightly increased by addition of 0.1 per cent methyl cellulose CPS 4000. This rapid agitation served also to maintain circulation of the nutrient fluid over the cell surface and to maintain adequate oxygenation of the cell and culture fluid. In the later study,[2] a Brunswick type shaker was used. This shaker could handle culture flasks up to 1.5 l. in size.

(2) As an extension of earlier studies on the influence of cell population densities on proliferation, it seemed probable that it would be necessary to start the culture with a high population cell density.

In exploratory experiments, cells of clone strain L-929 showed rapid cell proliferation under these experimental conditions. The present study gives additional data and development of the concepts and equipment on the growth of certain strains of fixed tissue cells in this type of agitated fluid suspension culture.

General Materials and Methods

Shaker, incubator, and culture flask design used. Cultures were planted in modified Erlenmeyer or modified flat-bottom boiling flasks and were shaken on a modified Brunswick Type AV shaker* (FIGURE 1). This gives the flasks a somewhat rotary motion, so that rapid agitation can be obtained with only minimal frothing.

From earlier results in the study of rate of rotation on roller tube cultures,[1] it was thought likely that the optimum rate of shaking would probably be slower than the minimum speed of the standard Brunswick AV shaker. Before placing the shaker in use, the motor drive was therefore changed by installing an additional countershaft and cone pulley, so that the minimum rate of revolution of the shaker was reduced approximately to 4600 rph. In studies described in the present paper, the slow range was found to be less efficient, so this slow speed drive will not be described in detail.

This shaker is usually sold with clamps to hold Erlenmeyer flasks. Exploratory studies showed that the cone shape of the Erlenmeyer flask tended to damp the swirling culture fluid to form a thin layer on the inner surface of the flask, and so reduced oxygenation of the culture. With the flat-bottom boiling flask, the shape of the flask was such that the fluid tended to form a thin layer of much larger area that extended some distance above the equator of the flask. Since the greater extent of this thin film seemed to be particularly advantageous for gaseous exchange, for all latter experiments with cultures of larger size, this flat-bottom spherical type boiling flask of 1 l. or 1.5 l. capacity was used. In order to mount these spherical flasks on the shaker, special clamps were obtained from the manufacturer (FIGURE 1-I).

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* New Brunswick Scientific Company, New Brunswick, N. J.

In pipetting culture fluid out of the usual flat-bottom flask, the vertical neck of the flask did not allow draining the flask by means of the pipette. The throats of all flasks were therefore bent at a slight angle so that, as a pipette entered the throat, its tip normally struck the junction between the flat bottom and the curved wall of the flask. This was found to allow practically complete drainage of the fluid from the flask. Still later in the study, when it was decided to have a continuous flow of gas through the flasks, this single throat was substituted by two throats near the top part of the flask, and each throat was sloped to this angle. These throats respectively allowed inlet and outflow of gas and change of culture fluid. The throat for the inlet of gas was drawn down to a tip of about 2 mm. ID inside the flask and was curved to lie parallel with the curvature of the flask. This was done in the hope of obtaining more efficient mixing of the incoming gas with that already in the flask.

A wooden case to serve as an incubator was fitted around this shaking unit, leaving the motor and countershaft assembly outside the case. The grease fittings of the shaker were extended through the wall of this case so that the shaker could be easily lubricated. This case was lined inside with 1-inch celotex for heat insulation. On the bottom of the case, just below the large drive pulley of the shaker, four 200-watt tubular electric lamps were mounted in two parallel series. These served as a heater for the incubator. The total heat output of these lamps was regulated by means of a variable transformer mounted outside the incubator and wired into the heater-lamp circuit. Temperature was further controlled by means of two metastatic mercury-in-glass thermoregulators,* each with its own supersensitive relay. These regulators were set to 37.5° and 38.0° C. respectively. To each of the four spokes of the large drive pulley of the shaker, situated below the platform, a 2-inch-wide vane of light sheet aluminum was bolted the entire length of the spoke. These vanes served to keep the air in the incubator in motion and prevented local overheating. Finally, the incubator was fitted with a chart type temperature recorder.†

Planting of cultures, transfer of cultures, and changes of culture fluid. Cultures from established strains of cells were planted as unsieved or sieved cell suspension[3] taken from T-30 or T-60 flasks[4] and made up to desired population density with fluid culture medium used. Various cell types were tried, including those of mouse fibroblast clone strains‡ L-929[5, 6] mouse liver epithelium

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* American Instrument Company, Silver Spring, Md.

† Foxboro Company, Foxboro, Mass. Chart No. 879069.

‡ When tested recently by reinjection into C_3H strain mice from which they were originally taken, both of these clone strains were able to give rise to tumors at the injection site in a very small percentage of those mice injected.[5, 6]

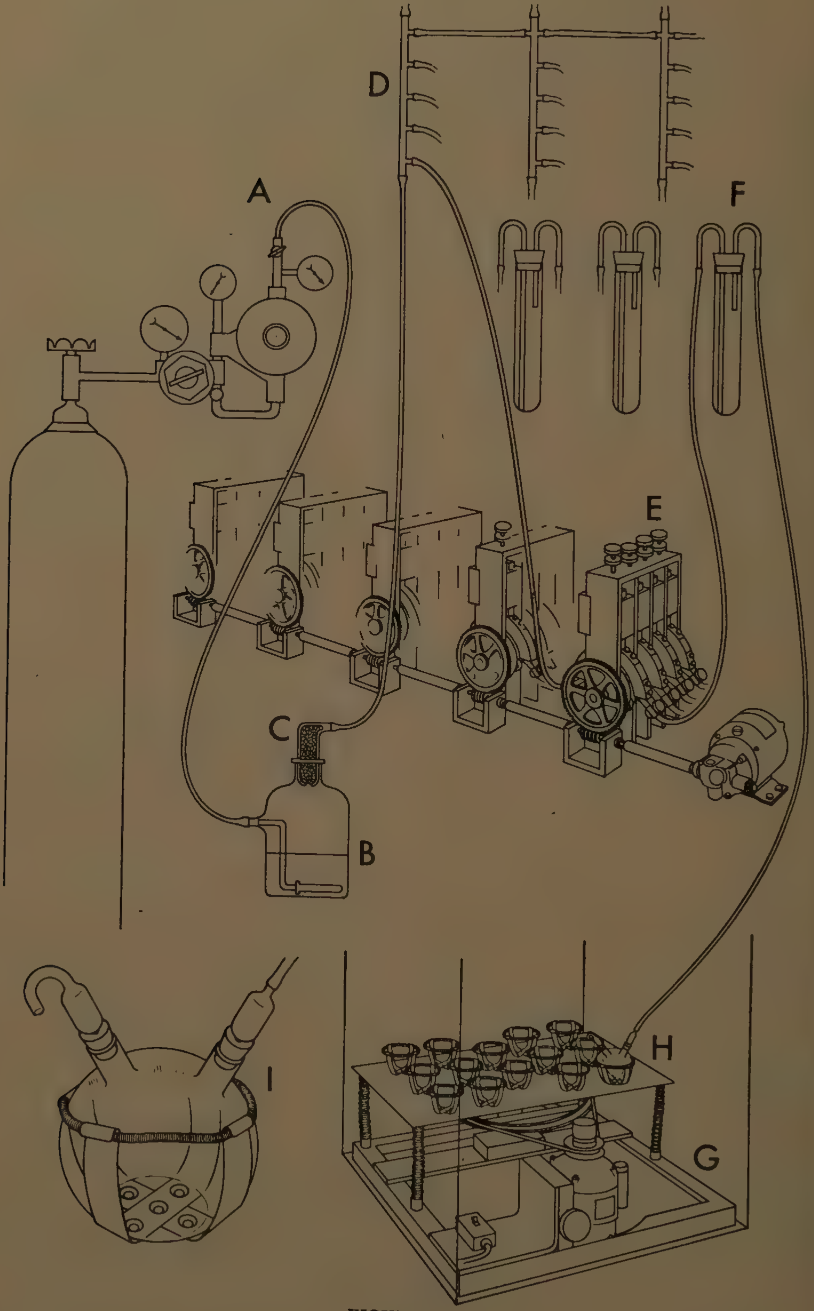

FIGURE 1.

1469,[6, 7, 8] and of the HeLa strain of human cervical carcinoma.[9, 10] In starting a shaker flask culture, the nutrient fluid volume was limited to keep cell population per unit volume of nutrient high. At consecutive nutrient fluid changes, the fluid volume was increased until, with a 1.5-liter flask, the densely populated culture would ultimately approximate 400 ml. total volume. All cultures were closed or subdivided at this volume or earlier.

Nutrient fluid changes were usually made thrice weekly. At each, the entire cell suspension was transferred to centrifuge flasks and centrifuged at 400 rpm. for approximately 30 minutes. Certain cell strains, notably the HeLa strain, have required stronger centrifugation, up to 900 rpm. for 30 minutes, to effect a clean separation of cells and nutrient, even when no methyl cellulose was used in the nutrient fluid. Following centrifugation, as much as possible of the supernatant fluid was pipetted off, and the centrifuge bottles were washed three times with small amounts of fresh nutrient fluids to prevent loss of cells. These washings were returned to the culture flask and then made up to correct final volume by additional nutrient fluid.

Culture media used. Several variations were made in the fluid media used. Media will be described for each study. Earle's balanced salt solution was used in all studies. In some studies, it was increased in dextrose content with corresponding reduction in sodium chloride to effect osmotic compensation. This concentration of dextrose in the diluting saline used in the later experiments was estimated to give 2.5 to 3.0 g. per liter in the culture media as actually used.

Gassing equipment and method. In early studies, at each change of culture nutrient fluid, the supernatant gas in the culture flask was flushed out with 5 per cent CO_2 in air. The flask was then sealed. The gas was not changed until the next fluid replacement, two to three days later. With cultures that contained relatively large amounts of cells, the pH shift to the acid side was extremely rapid after sealing the culture, and was so great as to retard growth and injure the cells. Shortly after the culture flask was unsealed, the pH drifted alkaline. It seemed obvious that the acid drift was largely due to CO_2 generated by the cells, while the alkaline drift resulted from loss of CO_2 on unsealing the culture. Further, instances were seen of culture necrosis that occurred in very large and dense cultures, possibly arising from exhaustion of oxygen in the culture flask. It was finally decided that to eliminate or better evaluate the influence of these complicating factors, it was necessary to have a constant, slow flow of gas through each culture flask. The mechanism worked out to accomplish this is shown in FIGURES 1 and 2. In experiments where continuous gassing was used, the rate was uniformly 140 ml. per hour.

In the earlier studies, where the cultures were continuously gassed, the

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FIGURE 1. (A) Tank of 5 per cent CO_2 in air. The tank was fitted with a Hoke (Hoke Incorporated, Englewood, N. J.) two-stage pressure reducing valve No. 901, with high and low pressure gauges, a Hoke No. 804 large-diaphragm very low pressure regulator, and 36-ounce range "ouncer" gauge at 16 ounces pressure. From here, the gas was saturated with water vapor by passing through a Selas (Selas Company of America, Philadelphia, Pa.) XF porosity ceramic filter in 2 per cent sulfuric acid (B). In this solution, the acid acted to reduce bacterial and mold growth. The saturated gas then passed through a column of glass helices to remove water droplets (C), and from there passed to a manifold (D) which distributed the gas to a multiple-unit metering device (E), set to allow each culture to receive 140 ml. of gas per hour. From each unit of this metering device, the gas passed through a bubbler tube (F), which indicated when the gas was flowing, and from thence it passed through a cotton-packed trap to the gas inlet tube of the culture flask, which was mounted on the shaker (G). The outlet tube of the flask was also protected by a cotton-packed trap (I).

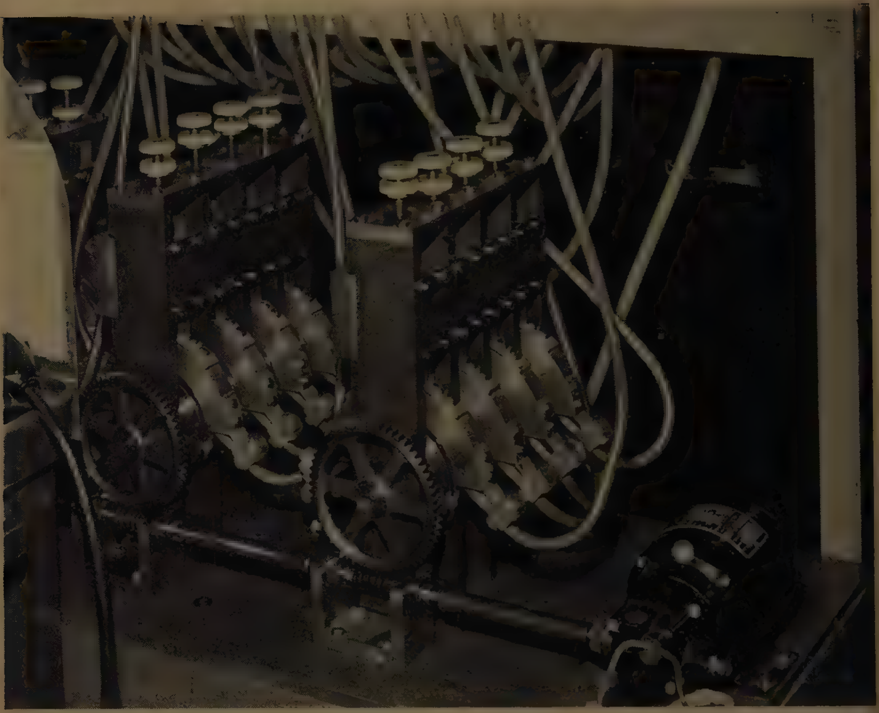

FIGURE 2. Close-up view of part of the gas metering device. This shows eight units of the device, each of which regulated the amount of gas fed into one culture. Each unit consisted of an anodized surgical rubber tube, 3/16″ bore, 1/16″ wall, which passed through the metering unit. The gas was forced forward through the tube of each unit by the rotation of a circular drum peripherally fitted with five equidistant rollers. With rotation of the drum, the rollers progressively pressed on the tubing and constricted the tube lumen by flattening the tube against the semicircular metallic upper plate. Spacing between rollers on the drum and the upper plate, and consequent degree of constriction of the rubber tube were controlled by adjustment screws at the top of the frame. Tubing was lubricated by Molykote Type Z (Alpha Corporation, Greenwich, Conn.).

number of cultures that showed infections was very high. This difficulty was finally traced down to bacteria growing through the cotton-packed gas filters guarding the gas inlet and outlet of the culture flask. This difficulty was largely eliminated when the gas inlet filter was routinely changed at each fluid renewal. The outlet filter was changed each third fluid change, that is, once a week.

Enumeration of nuclei. Methods of enumerating nuclei for following cell population change were substantially those previously described[11] except that, instead of sacrificing an individual culture for nuclei enumeration, an aliquot (usually 1 ml.) of the cell suspension was withdrawn from the culture while it was being shaken, and the nuclear enumeration was made on this. Since the HeLa cell suspensions tended to form aggregates, it was harder to get a true aliquot of the culture, and consequently, nuclear enumerations on this cell type were much more troublesome and less accurate than on the clone L-929 or the mouse liver clone 1469. These aggregates do break up in the citric acid fixation, however, so that the nuclei are clearly separated and easily counted.

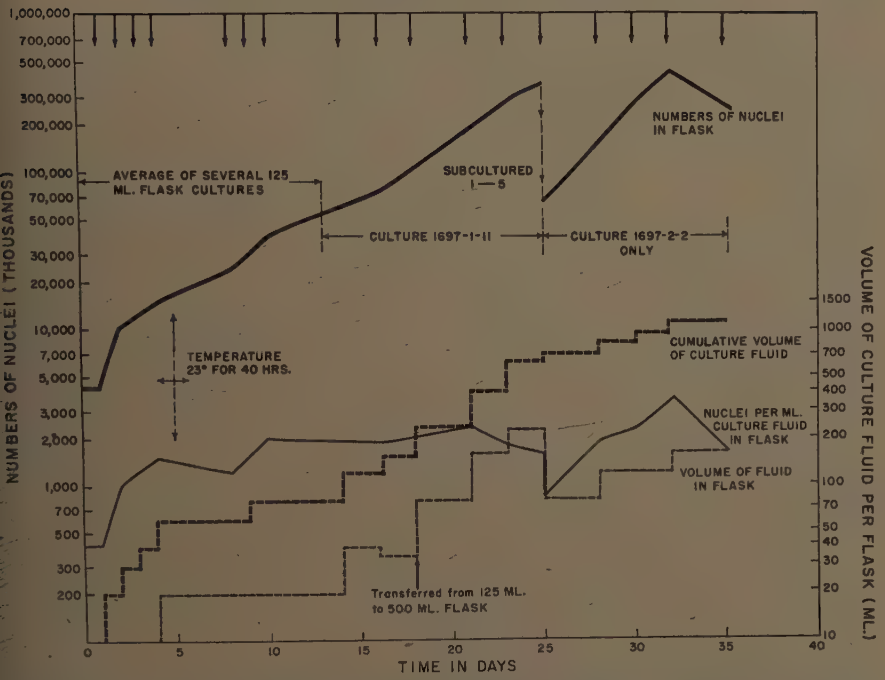

FIGURE 3. Summary of data from experiment 1697 on clone L-929 mouse cells. In this and subsequent figures numbers of nuclei in the culture (heavy solid line) and numbers of nuclei per milliliter of culture volume (light solid line) are shown along the left vertical axis (in thousands). Volume of nutrient in each culture (light broken lines), as well as the total cumulative volume of fluid used in each culture (heavy broken line) are shown along the right side vertical axis. Time in days is indicated along the lower edge of the figure. Each change in the nutrient is shown by a vertical down-pointed arrow along the top edge of the figure. Subdivision of cultures is indicated in text and in figures.

Results

A preliminary summary of certain data from the first experiment (No. 1697) was given[2] in a previous paper.* More detailed analysis is given in FIGURE 3 and in the text below. Clone strain L-929 fibroblasts were started in modified 125-milliliter Erlenmeyer culture flasks, using cells equivalent to 4,260,000 nuclei suspended in 10 ml. of fluid medium per culture. Nutrient fluid consisted of 30 per cent horse serum, 20 per cent 1:1 chick embryo extract, and 50 per cent balanced saline. To this fluid, 0.1 per cent methylcellulose was added. After the fourth day, glucose was raised to 2.0 g. per liter and was continued at approximately this level until the 35th day. Supernatant gas in each culture was replaced at each fluid change but cultures were not gassed between transfer intervals. Rate of shaking was 6000 rph.

At 18 days, one culture (1697-1-11) was transferred to a 500-milliliter Erlenmeyer flask and the fluid volume was increased to 80 ml. The fluid volume was progressively increased until, at 25 days, the 230 ml. of cell suspension contained cells equivalent to 362 million nuclei, while in order to obtain this amount of cells, the culture had required use of a total cumulative volume of 700 ml.

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* The present final calculations on data of this experiment superseded the earlier preliminary approximations reported.

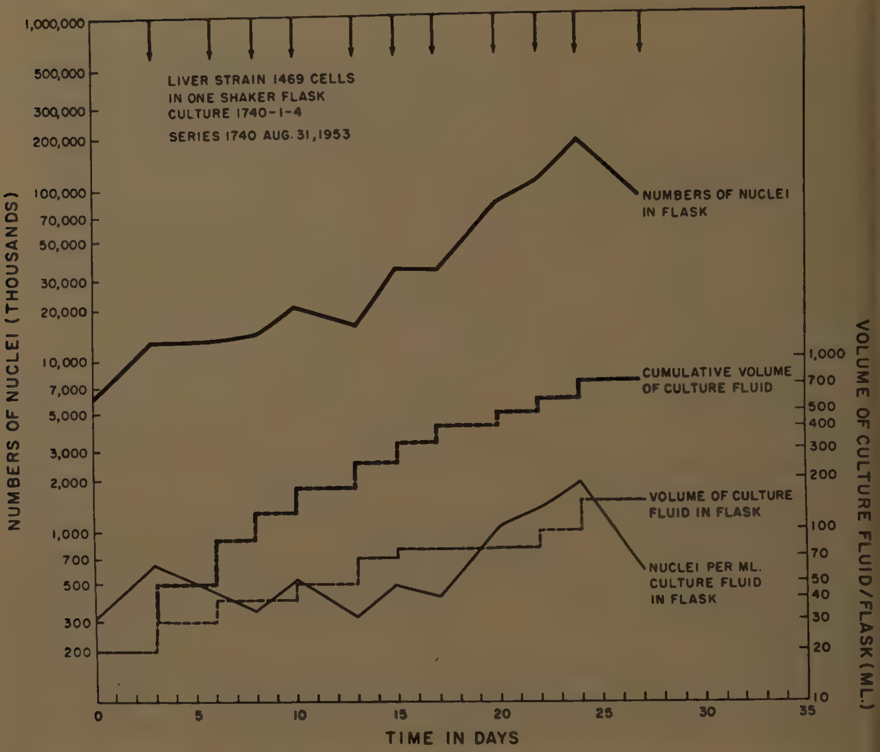

FIGURE 4. Summary of data from experiment No. 1740 on clone strain No. 1469 mouse liver epithelium.

of culture fluid. From data presented in an earlier paper,[5] 250 million cells of this strain are considered approximately 1 g. wet weight. On this basis, the culture contained in excess of 1.4 g. of cells wet weight.

At this time, the culture was divided and subinoculated into five 500-milliliter Erlenmeyer flasks. Four of these were closed for study. One (No. 1697-2-2) was continued for 10 days longer. At seven days after subinoculation, this culture contained 120 ml. of culture fluid and had a cell population that exceeded the equivalent of 430 million nuclei or 3.6 million nuclei per milliliter, while the total cumulative volume of culture fluid used on this culture generation up to its peak at seven days was only 320 ml. At seven days, the culture fluid was replaced as usual and its volume was increased to 100 ml. During the next three days, the culture dropped to a pH below 7.0. Examination of the cells showed general necrosis, obviously from overcrowding and exhaustion or toxication of the medium during the week end three-day transfer period. The culture was therefore closed.

Results from experiment No. 1740 are given in FIGURE 4 and in the text below. Cells used were those of clone strain No. 1469 mouse liver epithelium, first explanted from a two-day-old mouse on April 15, 1948. This clone strain grows luxuriantly in surface substrate cultures in the same horse serum-chick embryo extract fluid nutrient described in the preceding experiment. One change was made in the medium, however. In an earlier study with cells of this strain, it has been found that addition of methyl cellulose to the nutrient fluid gave such perfect suspension of the cells in the medium that they could not be centrifuged out even at high velocities. Methyl cellulose was therefore omitted from the fluid medium.

One culture was started in a 125-milliliter Erlenmeyer flask, which an inoculum of cells in suspension equivalent to 6.3 million nuclei and, in a volume of 20-ml. horse serum-chick embryo extract fluid nutrient. This was equivalent to 313 thousand nuclei per milliliter. Rate of agitation was 6000 rph. Cells were washed and fluid nutrient was changed thrice weekly, at which times the gas in the flask was also changed. On the 15th day, culture was transferred to a 500-milliliter Erlenmeyer flask. Volume of nutrient was gradually increased and reached 100 ml. between the 22nd and 24th day, with a total cumulative volume of 590 ml. of nutrient fluid. At the 24th day, the number of cells in the culture was equivalent to 192 million nuclei or 1.92 million nuclei per milliliter. On the 24th day, 150 ml. of fresh nutrient was substituted. During the three-day week end transfer interval, extensive necrosis of the culture supervened, apparently due to overcrowding. The culture was therefore closed.

Results and the analysis from culture No. 1846 are given in FIGURE 5 and in the text below. Cells of the HeLa strain of human carcinoma of the cervix of the uterus were cultivated on glass substrate cultures, removed from the glass by scraping, and then planted as a cell suspension at a cell population density equivalent to 6.8 million nuclei in 10 ml. of culture fluid; that is, 680 thousand per milliliter in each of two cultures, each in a 125-milliliter flask. Nutrient medium consisted of 30 per cent human serum, 20 per cent 1:1 chick embryo extract, and 50 per cent balanced saline, with 2.6 g. dextrose per liter of medium. Initial velocity of agitation was 7200 rph. Cultures were gassed at each fluid transfer.

Growth in this culture was different from that seen in cultures of other cell types. Many of the cells tended to grow in more or less spherical masses of up to 1-millimeter diameter. These larger sizes sometimes showed dark necrotic areas of small size in their center. Other smaller masses, all tending to spherical shapes, occurred, while many cells were entirely separate and independent. These also were approximately spherical in shape, but often had numerous short processes around their periphery.

At 40 days, an attempt was made to get rid of large cell aggregates by increasing the velocity of agitation of the culture to 12,600 rph. This higher velocity did reduce the larger cell aggregates both in number and size. Many spherical cell masses, however, did remain throughout the course of the experiment, although they were usually of smaller size. Many cells occurred as separate spherical individuals.

At 35 days, the culture was transferred to a 1000-milliliter flask and, from this time on, was continuously gassed. At 61 days, the population was equivalent to 433 million nuclei in a volume of 240 ml. or 1.8 million per milliliter.

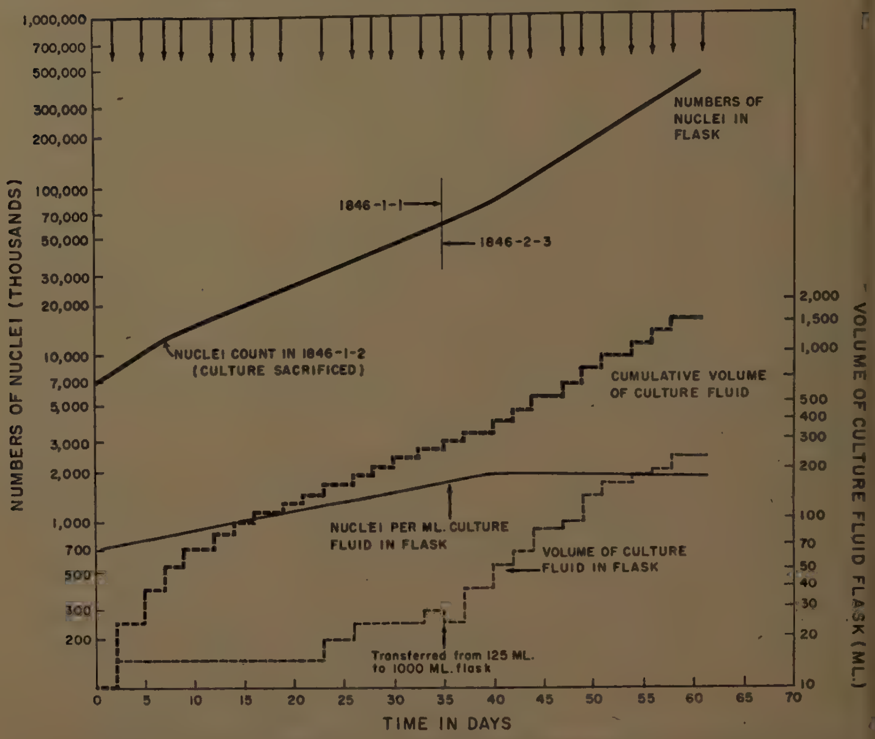

FIGURE 5. Summary of data from experiment No. 1846 on the HeLa strain of human carcinoma of the cervix.

Assuming the HeLa cell to be of the same diameter and density as the clone L-929 cell, this ultimate cell population would be about 1.7 grams wet weight, with a cumulative nutrient fluid consumption of 1560 ml.

Results and their analysis from culture No. 1912 are given in FIGURE 6 and in text below. Strain L-929 fibroblasts were started in the same horse serum-chick embryo extract medium used in the first experiment presented, and 0.1 per cent methyl cellulose was used. Dextrose was 2.6 g. per liter of final medium. Culture flasks used were 1-liter, flat-bottom, spherical. Original volume of fluid nutrient was 40 ml. and original inoculum equivalent to 24.6 million nuclei, or an average of 614 thousand nuclei per milliliter. Rate of shaking was 12,600 rph. The culture was continuously gassed. The cell population increased rapidly until, at 12 days, it was equivalent to 753 million nuclei, or more than 3.0 g. wet weight of cells. At this time, the culture contained 200 ml. of nutrient fluid, so that the population was equivalent to 3.76 million nuclei per milliliter and had a total cumulative volume of culture fluid of approximately 585 milliliters. The culture was subdivided at this point into five aliquots. Four of these were discontinued. The fifth (1912-2-3) was continued. This culture had an inoculum equivalent to about 151 million nuclei and an initial culture fluid volume of 175 milliliters. Within the next four days, the population of the culture exceeded that equivalent to a nuclear

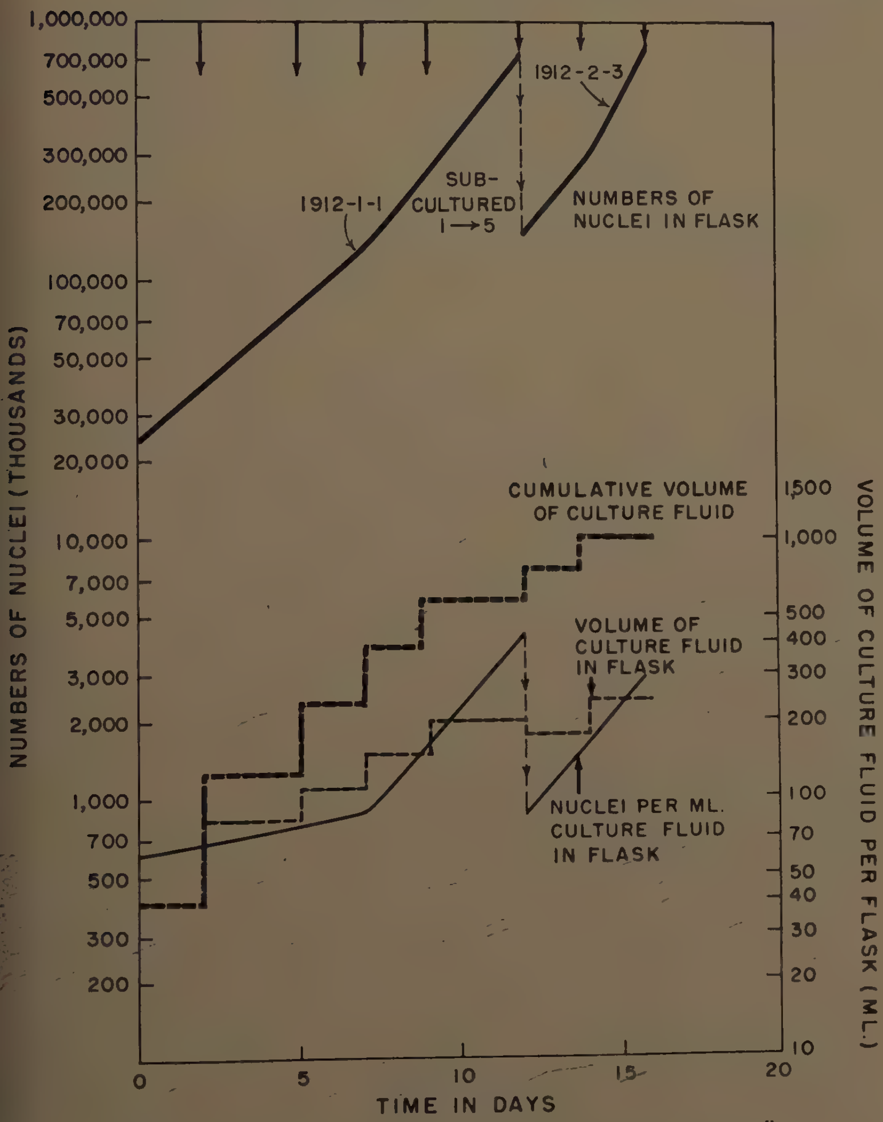

FIGURE 6. Summary of data from experiment No. 1912 on clone L-929 mouse cells.

count of 778 million, contained in a volume of 250 ml., or 3.1 million per milliliter. Total cumulative fluid volume for this transfer generation was only 325 ml. Cell population was estimated at 3.1 g. wet weight. The culture was finally closed due to bacterial infection.

Results and analyses from culture No. 1939 are given in FIGURE 7 and in text below. This culture was started from a HeLa cell suspension prepared by shaking and scraping glass substrate cultures. Initial inoculum in a 1.5-liter

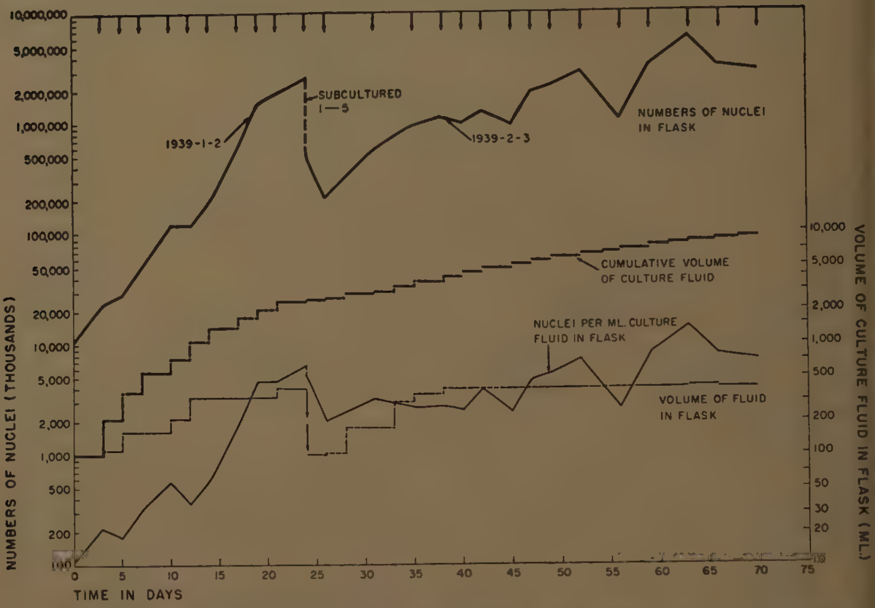

FIGURE 7. Summary of data from experiment No. 1939 on HeLa strain of human carcinoma of the cervix.

flask was equivalent to 10.8 million nuclei suspended in a volume of 100 ml. nutrient fluid made up of 30 per cent human serum, 20 per cent egg ultrafiltrate prepared from whole unincubated eggs* and 50 per cent balanced saline with dextrose at 2.6 g. per liter of final medium.† The culture was begun with 0.1 per cent methyl cellulose, but this was later discontinued due to difficulty in centrifuging the cells out. Gassing was continuous.

Increase of the cell population was rapid until, at 24 days, it was practically equivalent to 2.6 billion nuclei in 400 milliliters of nutrient fluid, or 6.5 million nuclei per milliliter. The total cumulative fluid volume was approximately 2500 ml. of nutrient fluid. This culture was subdivided into five flasks, each with an initial inoculum equivalent to 516 million cells in 100 ml. of fluid. During the next two days, the population of the culture from which an aliquot was taken dropped to 216 million nuclei, or 2 million nuclei per milliliter. Replacement of culture fluid was continued for a total of 68 days, or 43 days after subculture. During this period the population level continued to increase, although there were several short-term drops in the curve. These are probably due to our inability to keep the number of cells per milliliter of culture constant. The worst of these was at 56 days from the start of the study, just following a three-day week end, at which time there was obvious necrosis in the culture, probably due to exhaustion of the culture fluid or to a reduction of oxygen in the flask.

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* Studies on this whole egg ultrafiltrate are now being prepared for publication. The ultrafiltrate from whole unincubated eggs has been found to be fully the equal of chick-embryo extract for cell proliferation.

† Since the time of this study, a strain of HeLa cell has been adapted to where it grows luxuriantly in these fluid suspension cultures in a medium of 30 per cent horse serum, 20 per cent embryo extract or whole egg ultrafiltrate, and 50 per cent balanced salt solution with increased glucose.

Maximum population of the culture, as judged by nuclear counts of aliquots, was reached 40 days from explantation of the second generation and was approximately equivalent to 5.9 billion nuclei, in 420 ml. total culture volume, or 14 million nuclei per milliliter, and a total cumulative volume of 8 liters. Since 400 ml. was the practical fluid capacity of the 1.5-liter shaker flask, the volume was not increased after the first 14 days of the culture generation (except for this single fluid change period near the end of the curve). At 42 days, degeneration of the culture began to appear and to become progressively severe. In other words, with constant renewals of nutrient but with practically no actual increase in volume, this culture generation was maintained and reached its maximum population equivalent to 5.9 billion nuclei after 39 days.

Discussion

While no serious trouble has been experienced with frothing or adhesion of cells to the glass in this type culture, in sporadic cases trouble has been experienced with a fibrillar precipitate. The nature or cause of this is as yet unknown, although it is possibly associated with too low a cell population level in each unit volume of fluid.

The results presented from these three types of cells confirm earlier results presented in showing that a variety of cell types can be grown in fluid suspension cultures. From the successful results so far obtained, it appears probable that, as adequate nutrients are worked out, other cell types can be grown by closely similar or comparable procedures.

From the beginning of the study of cell suspension cultures, the use of methyl cellulose was considered a secondary factor, one that assisted in keeping the cells in suspension. With the liver epithelium and, to a less extent, with the isolated cells of the HeLa strain, however, its use definitely complicated the technical handling of the cultures by suspending the cells so perfectly that it became almost impossible to centrifuge them out at a reasonable velocity. It also interfered greatly with certain chemical studies of the cultures. The demonstration that the cells could be maintained in suspension without addition of methyl-cellulose or other agent to increase viscosity of the medium definitely increased the technical ease, rapidity, and accuracy of handling the cultures. With cell types and experimental conditions used, the cells can be held in suspension without difficulty merely by agitation of the culture medium. At the present time, our shaker is set at from 12,600 to 13,800 rph., or 210 to 230 per minute. Slower rates of agitation have given poorer growth. Whether a faster rate might be desirable has not been tested, since the present rate is about the maximum at which our shaker, with its added slow-speed shaft, can be continuously operated. Whether rates of agitation now used are optimal for other cell types must await further study.

Rapid agitation of the culture not only keeps the cells in suspension but helps keep them from clumping. It washes the nutrient fluid rapidly over the surface of the cell, keeps the culture fluid and the cells oxygenated by diffusion of oxygen from the overlying air in the culture flask, and allows excess CO_2 produced by the cells to diffuse into the overlying air in the culture flask. By gradually changing this overlying gas phase, fresh oxygen is continually brought into the culture, and excess CO_2 is carried away. This last helps to conserve the alkali reserve of the nutrient solution and helps to keep it from swinging too far acid.

Types of agitation of the medium other than that described have not been tested exhaustively because of difficulties that they showed in exploratory studies. The use of a reciprocating shaker and of a culture flask with baffles both resulted in the culture fluid being thrown as a spray and wetting the gas inlet and outlet filters, with resultant irregularities of gas flow and rapid bacterial contamination of the culture. Use of a magnetic stirrer was discarded due to complications that arose from the limited sizes and designs then available. With the type tried, a magnetic stirrer rod rolling with its entire length in contact with a flat-bottom container definitely acted like a ball mill and ground cells up. The magnetic stirrer might be successfully used by designing a special stirrer rod that had very small surface in contact with the floor of the flask. An external propeller type stirrer appeared technically more difficult than did the shaker with the cultures of the present sizes, and it was not tested.

An attempt was made to obtain both shaking and oxygenation of the culture by bubbling the mixture of 5 per cent CO_2 and 20 per cent O_2 through a diffusion disc and up through the fluid culture medium. This caused so much stable froth formation that an antifoam agent would have been necessary. Since addition of an antifoam agent would have complicated the experimental conditions, this has not been studied further. With very large size cultures or with pilot plant installations, this use of an external agitator or the passage of gas through the nutrient and the use of an antifoam agent must be re-explored.

With these shaker-type cultures, if a continuous gassing equipment is not available, cultures that contain many million cells may be built up by flushing out each culture flask once a day or, better, twice a day with the gas mixture used. As the population gets more dense, however, as with the culture shown in FIGURE 7, a continuous gassing device is probably necessary. Even with our present gassing equipment, the volume of gas delivered to such a culture is inadequate. Plans are already being drawn up to increase this gas delivery rate greatly.

Some idea of the amount of O_2 necessary may be obtained from the following rough estimates. With the strain L cell, assuming a specific gravity of one, 250 million cells would be about 1 g. wet weight. At 37° C., assuming a QO_2 of 10, this amount of cells would take up a ml. of oxygen per hour. If we assume the average diameter of the HeLa cell as comparable to that of strain L, with its 5.9 billion cells (FIGURE 7) would contain approximately 23.6 g. of cells, wet weight, and would take up 23.6 ml. of O_2 per hour. This would be equivalent to the oxygen in 118 ml. of air that must reach the cells each hour in order to allow efficient aerobic metabolism.

At present, two difficulties in particular interfere greatly with ease of maintenance of this type of fluid suspension culture. These are (1) difficulties associated with having to centrifuge the cells out at each fluid transfer; and

(2) difficulties associated with keeping the cell population in constant balance with the volume of nutrient fluid in the culture flask. This last arises from the fact that, with present facilities, it has been impossible to get nuclear counts through rapidly enough to keep pace with the renewal of the culture fluid. As a result, estimates of what the correct volume of newly renewed culture fluid should be have been based on microscopic examination and on mere visual inspection of the turbidity, the actual nuclear counts being completed 24 to 96 hours after fluid transfer. Attempts are now being made to correct these primary difficulties. When this type culture is correctly maintained, however, as in culture No. 1912, its efficiency is extraordinarily high and, certainly, much higher than that of any of the more conventional types of tissue cultures. Thus, in culture No. 1912 of clone L cells in the second generation, starting with an inoculum of about 150 million cells, the population increased in excess of 725 million within four days, a net increase of over 575 million cells, or over 2 g. estimated wet weight. This gain was obtained with use of a total expenditure of only 325 ml. of nutrient fluid medium. Of this volume, 50 per cent was the balanced saline, so that the volume of serum and undiluted embryo extract used was approximately 162.5 ml.

For efficient usage of this type of culture for the production of relatively large numbers of cells in batch lots, it is best, from our experience, to build the culture up to practically maximal volume and a dense population level. In culture No. 1939, for instance, at 24 days, 8 g. of cells could have been taken for experimental work. After adding fresh nutrient, 2.4 g. could have been used to maintain the cultures in rapid proliferation.

Without question, the methods, equipment, and technology so far developed for handling such relatively large cultures of animal tissue cells represent only an exploratory study. Due to limitations of time, no consideration has been given to certain recent applications of this method of culture, to chemical studies with the cultures, to continuous methods of feeding, and to recent attempts to simplify the feeding and harvesting of the cultures. One study may be mentioned, however. As reported in another paper now being prepared for publication from our Section,[12] we have recently been able to build up a nutrient solution, chemically defined and free from protein and higher protein cleavage components. In this solution, good continuous proliferation of clone L cells is being obtained, and has already continued for more than 120 days.

Work is now in progress on attempts to use this chemically defined protein-free medium in these fluid suspension cultures of the type we have described.

The results so far obtained with growth of animal tissue cells in progressively larger amounts further confirms our concept that, as media and technology are worked out, such fluid suspension cultures of animal tissue cells can be maintained in larger and larger size, and with progressively increased facility. As adequate culture media and mechnical facilities are worked out and become available, and as reason for large scale growth of animal tissue cells arises, there seems no reason that such cells should not be grown in large-scale commercial tank installations. Already the factors involved in the growth of the animal tissue cells in fluid suspension cultures impress us by their similarity to the factors involved in the growth of fungi in pilot plant and commercial installations of the antibiotics industry.

Summary and Abstract

More extensive and complete data are given on the growth of fixed tissue cells in fluid suspensions continuously agitated on a Brunswick shaker at speeds up to 13,600 rph. Cell strains used were clone L 929, clone 1469 liver epithelium, and the HeLa strain cervical carcinoma. An initial heavy cell suspension inoculum was necessary. All cell strains grew luxuriantly.

Maximum volume of any culture was approximately 400 ml. contained in a 1.5-liter flask. With the HeLa cell, an ultimate population of 5.9 billion cells, estimated at 23.6 g. wet weight was obtained. With such culture, special methods must be used to insure adequate oxygenation of the culture.

Equipment and methods used are detailed, and major difficulties are described. The method of culture is applicable to at least certain other cell types. It appears that the factors involved are such that the fluid suspension culture of animal tissue cells can be extrapolated to much larger scale and, ultimately, even to pilot plant or commercial size installations.

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Discussion of the Paper

Doctor Robert N. Hull (*Lilly Research Laboratories, Indianapolis, Ind.*): Doctor Earle has remarked that the method of growing cells in a fluid suspension developed by him and his co-workers at the National Cancer Institute at Bethesda, Md., is still in the experimental stage. Be this as it may, its significance is readily apparent, and its refinement will considerably project the future applications of tissue culture in research and in industrial production procedures.

In discussing his paper, I shall consider it from two points of view, namely: from that of tissue culture, and in respect to ascites tumor cells, inasmuch as the conference on which this monograph is based was organized for the discussion of these cells as research tools.

The first report submitted by Doctor Earle and his group a year ago, describing the growth of tumor cells in fluid suspension, marked a milestone in the field of tissue culture. For years, the technic of cell cultivation has been limited to relatively small size cultures, due to the conception that cells should be embedded in some sort of matrix, such as a plasma clot, and that they should be anchored to some solid substrate in order to undergo the process of cell division. Doctor Earle and his co-workers, as well as others, have disproved the need for plasma clots and have thereby increased the facilities of handling cells as well as of increasing the area of growth in two dimensional cultures. Now, with the development of the fluid culture, the possible size of cell cultures has been still further increased, and it has been demonstrated that cells can divide without being attached to a substrate.

Doctor Earle has made it a point to talk about the efficiency of the fluid culture in respect to the total volume of cells produced in a single flask. If, however, one examines the efficiency on the basis of the number of cells per milliliter of culture fluid, it is apparent in the first generation that the efficiency of this fluid culture is somewhat comparable to that obtained in conventional culture flasks. In the second generation, however, after a large population has been built up, the efficiency of the fluid culture then surpasses that of the two dimension flask cultures. Referring to Figure 6, showing the growth curve of culture No. 1912 (L-929), it is seen in the second generation that 150 million cells were increased to 750 million in four days' incubation, utilizing 250 ml. of culture medium. In terms of T-60 flask cultures of this same cell strain, this would be comparable to attempting to expand five completely filled T-60 flasks to 25 flasks. It would be possible to make this expansion, but certainly not in four days time nor without additional changes of medium. It would require several times the 250 ml. of medium expended in the fluid culture. Doctor Earle has pointed out that most of the experiments described in his presentation were not done with maximal proliferation in mind, but it is hoped that further study will result in the development of techniques that will provide even greater cell densities. It is interesting to note that this early attempt to grow tissue cells in fluid culture is quite inefficient in respect to the growth of ascites tumor cells in the mouse, since Doctor Klein has stated that densities of one billion cells per milliliter can be obtained.

Further possibilities for use of this fluid culture are many. At present, until further knowledge is acquired as to the nature or properties of cells grown under these conditions, it would seem that the most advantageous use of this method would be for the preparation of large lots of inocula for seeding roller tubes or other conventional flasks. These subcultures then could be used for the numerous types of investigation being carried out in tissue cultures. The fluid culture, further, makes possible the preparation of gram-size quantities of cells for chemical analysis or for extraction of specific substances. There is also the likelihood of utilizing cells in the shaker flask for experimental or production purposes. Although it has not yet been studied, there is the possibility that viruses could be grown directly in the fluid culture. Another rather remote possibility for a commercial application would be the growth of glandular cells in such a culture from which the secretory product might be recovered.

In respect to much of the work that has been done, or is being done, with ascites tumor cells, this method of tissue culture could be a considerable asset to this work. For example, many types of tumors have resisted the transformation to the ascites type and therefore can not be studied under the same conditions as those that have been adapted to fluid growth *in vivo*. It is possible, then, that such tumor cells could be grown in tissue culture in shaker flasks under conditions that would yield quantities of cells somewhat approaching that of the mouse ascites. Another service that the fluid culture could supply to the worker using ascites tumor cells would be that of normal cells serving as controls to the tumor cells, since there is not available *in vivo* any such source of normal cells in suspension other than those of the blood vascular system.

We have made some attempts to cultivate ascites tumor cells taken directly from the mouse in the shaker type culture. Doctor Irving S. Johnson, of our laboratory, has worked with the Ehrlich and sarcoma 180 ascites tumor cells, as well as with a third chemically induced tumor. None of these has given evidence of growth, but various degrees of survival have been noted. The sarcoma 180 cells have survived for the longest periods, which have been for more than a week. Sarcoma 180 cells removed from the shaker flask after six days and inoculated into mice produced an ascites in about 50 per cent of the animals inoculated. These initial experiments, although not successful, do not in any way preclude the possibility of growing ascites tumor cells in the new fluid suspension type of culture described by Doctor Earle.

IMMUNOSELECTION OF POLYPLOIDS FROM PREDOMINANTLY DIPLOID CELL POPULATIONS*

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Analysis of desoxyribose nucleic acid content and confirmatory chromosome counts in mouse tumors[1, 2] have led to the recognition of two major ploidy classes among neoplasms. Malignant cell populations are complex chromosomal mosaics, some of which are narrowly distributed around the diploid mode of 40, while others belong in the tetraploid category.

These nuclear constitutions have gained immunologic significance through their apparent influence on interstrain compatibility of a representative test material comprising 17 mouse ascites tumors.[3, 4] Transplantability or regression in mice with nonmatching *H-2* antigens (TABLE 1) indicates a functional relationship between the modal chromosome number and the immunogenetic dependence of homografts.

Diploid tumors, transplanted within the species but outside the isoantigenic type they have inherited from their primary source, fail to grow progressively. Deviating chromosome numbers facilitate more or less indiscriminate compatibilities. Some heteroploid neoplastic cells may be so unbalanced genetically that they are doomed to extinction. In many hyperdiploid and near-tetraploid cells, however, viability is unimpaired. These cells are able to grow progressively as homografts and to kill certain host genotypes that would be refractory toward comparable diploid grafts.

The attainment of antigenic autonomy is apparently independent of the tumor's histology, genetic derivation, length of serial passage, and virulence. Lymphomas and carcinomas of quite recent origin, for example the L No. 2 and TA3 ascites, may be extremely virulent for mice of the home strain—in this case the A-strain, but they cannot "take" across isoimmune barriers.

Most diploid tumors contain exceptional polyploid cells that result from endomitotic reduplication.[2, 5] These near-tetraploid elements normally constitute about 5 per cent of the neoplastic population. Their proportion tends to remain constant during prolonged serial passage in the genotype of origin. Occasionally and unpredictably, they rise to predominance, displacing the diploid cells down to or even below the 5 per cent frequency.

The latter type of shift has occurred spontaneously in our TA3 ascites carcinoma and was accompanied by loss of the tumor's formerly rigid host-specificity.[6] The A-strain carcinoma S3A[7] and a methylcholanthrene-induced C3H sarcoma followed through its developmental history by Sachs and Gallily[8] provide similar examples of increased host range in the wake of polyploidization. The S3A ascites changed its strain-specific diploid character to an aneuploid, widely transplantable condition (72 chromosomes) less than a year after its origin. Sachs's observations on his C3H sarcoma also cover a rela-

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TABLE 1

RELATIONSHIP BETWEEN CHROMOSOME NUMBER AND HISTOCOMPATIBILITY

Mouse tumor		Strain of origin and *H-2* genotype		Modal chromosome number	Lethal takes across H-barriers					
					A $H\text{-}2^a$	DBA2 $H\text{-}2^d$	C3H $H\text{-}2^k$	C57BL, 129 $H\text{-}2^b$	DBA1 $H\text{-}2^q$	Swiss $H\text{-}2^s$
TA3	ca.	A♀	$H\text{-}2^{a}$*	40	+	—	—	—	n.t.	—
6C3HED	ly.	C3H♀	$H\text{-}2^k$	40	—	—	+	—	—	—
A2	ly.	A♀	$H\text{-}2^a$	40	+	—	—	—	—	—
DBA	ly.	DBA2♀	$H\text{-}2^d$	42	—	+	—	—	—	—
Ehrlich	ca.	Het.	?	45–46	+	+	+	+	+	+
C1300**	nb.	A	$H\text{-}2^a$	66–70	+	+	—	—	+	n.t.
15091a	ca.	A	$H\text{-}2^a$	80±	+	+	—	—	+	—
TA32	ca.	A♀	$H\text{-}2^a$	74	+	+	+	+	+	+
A1	ly.	A♀	$H\text{-}2^a$	80±	+	+	—	—	+	—
MC1M	sa.	C3H♂	$H\text{-}2^k$	65–70	+	—	+	+	+	+
Ehrlich	ca.	Het.	?	76	+	+	+	+	+	+
Krebs 2	ca.	Het.	?	75	+	+	+	+	+	+
S37	sa.	Het.	?	40 and 80±	+	+	+	+	+	+

* $H\text{-}2^a$ corresponds to the old nomenclature $H\text{-}2^{dk}$.

** The chromosome numbers for C1300 are based on unpublished data of Doctor A. Levan.

Abbreviations: carcinoma (ca); lymphoma (ly); sarcoma (sa); neuroblastoma (nb); heterogeneous stock (Het).; + = lethal takes; — = regression; n.t. = not tested. Sex of animal in which tumor originated is indicated where known.

tively brief period. A prolonged serial transplantation history, therefore, is not a necessary prerequisite for reduced specificity.[9]

The manner in which the gene-controlled isoantigenic properties of cells are altered when the chromosome set becomes unbalanced is the subject of the present inquiry. It is as yet unknown whether (1) significant deviations from the normal diploid chromosome number of 40 in mouse cancer cells are regularly accompanied by a loss of effective cellular antigens,[3, 4, 6] or (2) whether transplantation into refractory hosts succeeds despite continued presence of *H*-antigens in the heteroploid grafts and corresponding antibody in the host. A third hypothesis assumes that increased production of antigens by polyploid cells paralyzes the immunological response of the host or neutralizes the acting antibodies before they are able to inhibit the implant.[9]

If the principle of the interrelationship between chromosome number and histocompatibility has general validity, immunologically hostile environments should exert selective pressure on mixed populations of diploid and heteroploid cells. The latter would have growth advantages. Balanced polyploids, present in small numbers in all the tumors examined, do not appear to have positive selection value. Genetically unbalanced chromosome sets, however, can crowd out the predominant diploid cell types under adverse transfer conditions. Shifts thus induced in the population equilibrium of a neoplasm have been tentatively interpreted as "immunoselection."[3, 10]

The experiments reported here were designed to scrutinize the correctness of the immunoselection concept through integrated cytologic, immunogenetic, and serologic techniques. Ideally, such an investigation requires comparison of various lines of a strain-specific tumor during prolonged periods of growth in the corresponding inbred mouse strain of origin and under isoantigenic handicaps due to known genetic differences at the *H-2* locus. The ascites-form of the 6C3HED lymphosarcoma was considered the most suitable material for our tests, since its main line adhered to a near-diploid chromosome constitution and to specific host requirements over the last four years.

Materials and Methods

(1) *Tumor and sublines.* The 6C3HED lymphosarcoma, estrogen-induced by Gardner in the thymus of a female C3H/St. mouse in 1941,[11] has been propagated as a solid subcutaneous tumor at the Jackson Memorial Laboratory since 1943, and was converted into the ascites form by G. Klein in 1950.[1] We have transplanted it serially as an ascites in C3H/St. mice since May 1951. For 202 weekly intraperitoneal passages through 940 C3H female mice aged two to three months, the lymphoma was invariably lethal.

After inoculation of large cell-numbers (average 2×10^8) into mice of inbred strains A/HA, C57BL/6, 129/Rr Jax, DBA/2, DBA/1 and into noninbred mice of the albino ICR Swiss stock, good temporary growth of the 6C3HED ascites, lasting for about 10 days, was regularly followed by regression. The refractory host genotypes are *H-2a*, *H-2b*, *H-2d*, *H-2q*, *H-2s*, while the compatible C3H/St. mice are *H-2k* (TABLE 1). C3H and the closely related strains CBA (*H-2*^{k}), CHI (presumably *H-2*^{k}),* which also are susceptible to the 6C3HED tumor, were derived by Strong from the common ancestral "C" stock.[12, 13]

The term "strain specificity," thus, is somewhat inadequate for the purposes of the present inquiry and should be interpreted as iso-antigenic specificity requiring known histocompatibility factors. The diploid form of the 6C3HED ascites lymphosarcoma is rigidly *H-2k* specific.

Since the malignant C3H lymphocytes appeared to persist longer and grow better in the Swiss stock (*H-2s*) than in the other foreign strains, a serial transfer line in Swiss was started in July 1952 from the 56th C3H main-line passage and, thereafter, was "nursed along" for 84 consectuive weekly transfers under conditions of delayed regression in Swiss mice. During this long sojourn in a foreign genotype, the cytologic and immunogenetic properties of the Swiss ascites subline were intermittently compared with those of the C3H/St. main line.

C3H/St. × DBA/2 resistant back-cross hybrids were used for the evaluation of gene-controlled isoantigenic changes. It was from one of these back-cross hybrids that the Swiss-adapted subline was recovered and established in DBA/2 (*H-2d*) mice, after the 84th serial Swiss passage had failed in June 1954.† A third, highly altered branch of the 6C3HED lymphosarcoma has been carried through nine passages in DBA/2 mice since then. The histories of the three lines are traced in FIGURE 1.

(2) *Mice.* All the animals used in this investigation were reared at our mouse colonies in Philadelphia and, later, in Buffalo, on the Morris formula diet.[14] C3H/St., ICR Swiss, and DBA/2 females, two to three months old,

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* According to recent unpublished tests of D. B. Amos.

† A possible explanation for this failure is given in the *Discussion*.

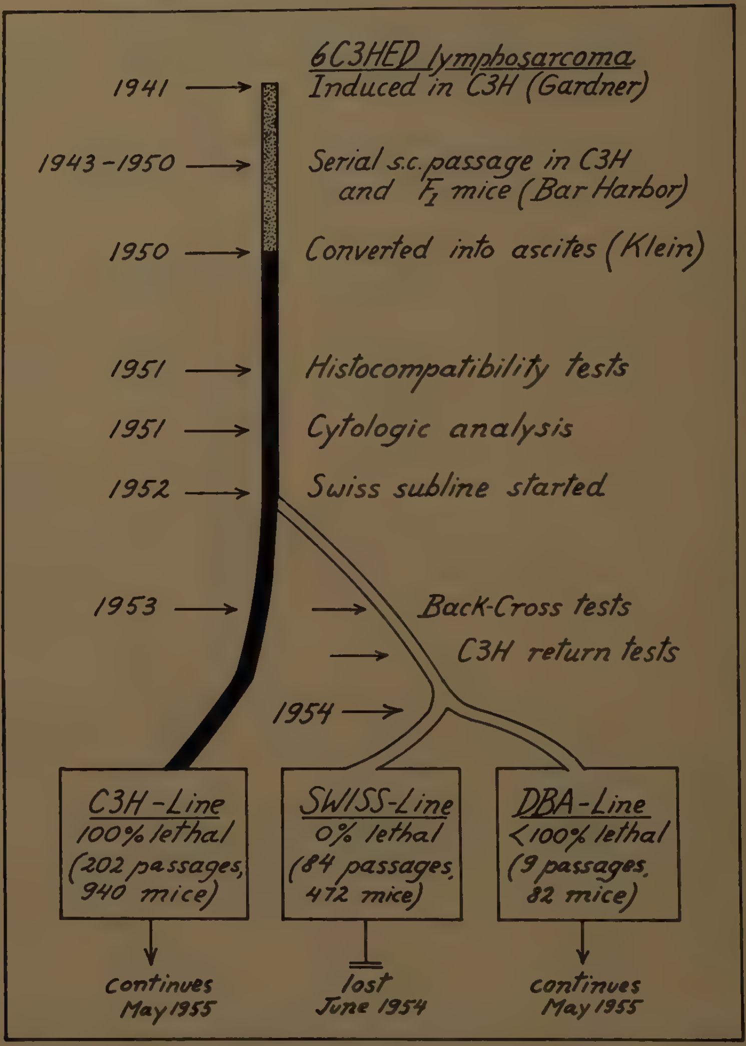

FIGURE 1. History of the serial transplantation of the 6C3HED mouse lymphosarcoma, and derivation of three sublines.

served for the propagation of the three separate tumor lines. The histocompatibility tests were carried out with young adult (C3H/St. × DBA/2)F_1 × DBA/2 resistant back-cross mice of both sexes.

(3) *Cell study.* Cell counts of the tumor inocula were made in a Spencer bright-line hemocytometer. For these counts, fresh tappings were diluted 1:100 with mammalian Ringer solution. The most convenient and objective method for measuring the distribution of cell diameters in living, noncompressed cells was to photograph suspensions immediately after introducing them into

the counting chamber. Diameters were then measured accurately on enlarged projections from the film negatives. This procedure eliminated error due to cell shrinkage during slow, tedious measurements carried out directly on the living cells.

For cytologic study, fresh ascites material was fixed and stained with aceto-orcein (1.5 per cent Gurr's orcein in 65 per cent acetic acid) and squashed on siliconed slides by thumb-pressure on a nonsiliconed coverslip.[2, 5] Representative samples from various transfer generations were surveyed for relative frequency of near-diploid (2n = 40 in the mouse) and polyploid (80±) metaphases, 100 or more well-spread chromosome plates constituting the usual sample. Exact chromosome counts were made whenever unusually well-spread, intact metaphase plates were encountered. Since lymphocytes have far smaller cytoplasms than some of the cytologically more favorable ascites tumors of the Ehrlich and Krebs-2 types, large-scale accurate counting was not feasible. This shortcoming, however, is compensated for by gross ploidy estimates on large numbers of metaphases and by comparative determinations of cell volumes.

(4) *Serologic tests.* The isoantigenic properties of the malignant 6C3HED cells of the three sublines were studied with antisera kindly furnished by Doctor Gorer's laboratory as follows: C57BL anti C3H = anti CK; C57BL anti A = anti CDK; Balb C anti C3H and anti A = anti EK. References to this nomenclature may be found in papers by Amos *et al.*[15] and by Hoecker *et al.*[16] Hemagglutinating titers, enhanced by the dextran method of Gorer and Mikulska,[17] were tested against C3H/St., DBA/2, DBA/1, and A/St. erythrocytes both before absorption and after absorption with equal volumes of packed lymphosarcoma cells. Antiserum absorbed with perfused normal C3H liver served as control. It was possible to carry out a simultaneous serologic analysis of samples tapped during the last four years. 6C3HED ascites from representative earlier transfers had been stored in our tissue-bank at −40° to −50° C. Some of the frozen material was still viable after 17 months of storage, but even the nonviable older samples were useful, having retained their full potency as absorbing antigens.

Results

(1) *Changes in virulence during propagation of 6C3HED lymphosarcoma in refractory hosts.* The 6C3HED ascites was uniformly virulent as long as it was carried in the C3H/St. strain of origin. During 202 weekly passages in C3H/St. female mice, it was always lethal. Mean survival time of 940 mice in this series was 11.4 ± 0.1 days (standard deviation 1.8) after a standard inoculum of 80 million cells.

The much lower dose of 2000 lymphosarcoma cells per adult C3H female mouse was also fatal without exception. Survival in this group was 20.9 ± 0.6 days (standard deviation 3.9). When only 15 cells were inoculated into each of 20 adult C3H/St. females, 19 died with typical ascites in 26.9 ± 1.4 days (standard deviation 6.1). No takes were obtained, however, in 20 tries with single cells.[6]

TABLE 2

DECREASED VIRULENCE OF THE 6C3HED LYMPHOSARCOMA ASCITES FOR C3H MICE AFTER PASSAGE THROUGH REFRACTORY MICE

6C3HED Ascites from	Injected intraperitoneally into (host strain)	Cell dosage	Total mice	Percent lethal takes	Mean survival (days)
C3H passage.....	C3H/St.	8×10^7	940	100	11.4 ± 0.1*
Gen. 1–202.......	Swiss, A, C58, DBA/1, DBA/2, 129	2×10^8	86	0	indefinite
Swiss passage.....	ICR Swiss	2×10^8	472	0	indefinite
Gen. 18–23.......	C3H/St.	2×10^8	25	100	33.9 ± 1.3*
Gen. 66–69.......	C3H/St.	2×10^8	43	97.4	79.2 ± 3.8*
DBA/2 passage...	DBA/2	2×10^8	82	<100	188.2
Gen. 1...........	C3H/St.	2×10^8	12	100	64.5

* D/SE for lines 1 and 4 = 17.3. D/SE for lines 1 and 5 = 17.8. p = <0.00001; the decrease in virulence is highly significant.

In TABLE 2, comparative survival data are summarized for the two attenuated 6C3HED sublines in Swiss and DBA/2 passage. Swiss mice were refractory, but permitted excellent temporary growth, with accumulation of 5 or more cc. of cell-rich, blood-free ascites fluid in 8 or 10 days after inoculation of 200 million cells. Complete regression occurred during the third week, and no recurrence was noted for one to eight months thereafter.

When an overwhelming dosage of 200 million Swiss-adapted cells was returned into compatible C3H/St. mice after 4 to 16 months of incompatible passage, host mortality was still near 100 per cent, but survival was now prolonged 3 to 7 times (TABLE 2).

A more sensitive gauge of declining virulence is provided by inoculation of low cell dosages (200 to 1200 cells) into susceptible infant mice. Such an experiment was performed with two- to three-day-old C3H/St. and (C3H/St. × Swiss)F_1 mice (TABLE 3). The latter are theoretically 100 per cent susceptible and should have had the additional advantage of carrying those compatibility genes toward which the Swiss-adapted tumor was being selected. It was, therefore, surprising to find the F_1 hybrid infants less susceptible than the C3H pups, not only to the virulent main line, but also to the attenuated Swiss subline of 6C3HED.

After the 84th serial passage in Swiss had failed to produce ascites, the subline was fortunately recovered from a DBA/2 mouse which, four months earlier, had received the Swiss ascites via a (C3H/St. × DBA/2)F_1 × DBA/2 backcross animal in a genetic test. In this exceptional animal (the only take among 32 DBA/2 females inoculated), the tumor had grown and persisted from February to June 1954, permitting subinoculation of further DBA/2 mice for nine serial passages. The majority of these DBA hosts developed enormous distensions (average 23.6 cc. of fluid) without otherwise showing serious debility. Their exudates abounded in viable lymphoma cells. Mitotic index was checked by repeated biopsies and was found to fluctuate between 1 and 3 per cent. This surprisingly high value seems incongruous with the characteristic low

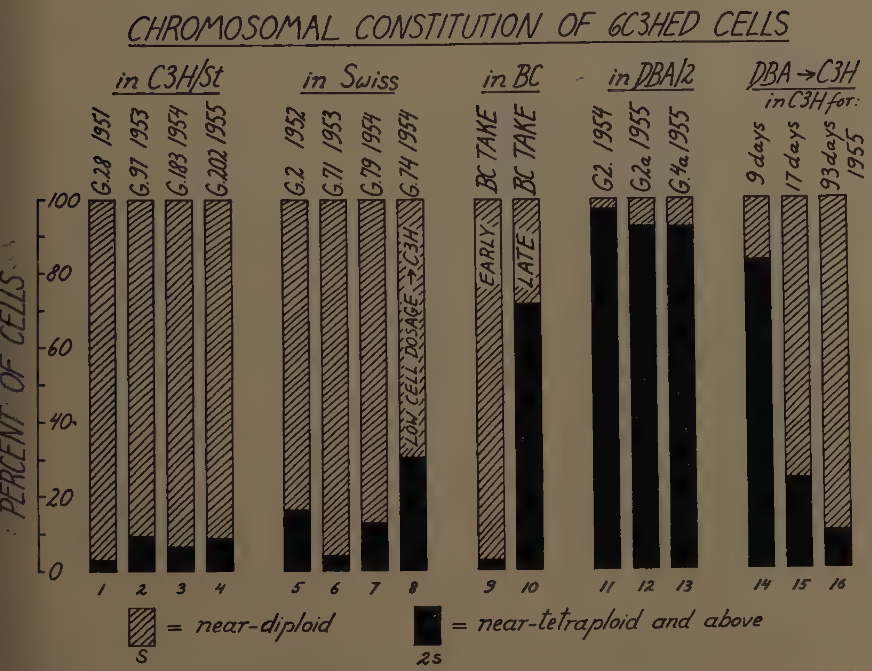

FIGURE 2. Percentage of near-diploid and near-tetraploid cells in various genotypes of mice and during several stages on the development of each 6C3HED subline.

virulence, but is compensated for by a quota of necrotic cells, suggesting an efficient balance between tumor growth and dying cancer tissue. The DBA/2-adapted line is the least "malignant" ascites tumor on record. Only six of the 82 DBA/2 mice inoculated with 6C3HED have died as of May 1955. In many of them, the lymphoma is still growing eight months after inoculation and, in a few, there has been complete regression.

The ascites in DBA-passage was generally blood-free and less metastatic than the original line in C3H/St. The latter has always given rather small amounts of very bloody exudate (about 3 cc.). Its invasiveness can be judged

TABLE 3

MORTALITY AFTER INOCULATION OF SMALL CELL NUMBERS OF 6C3HED ASCITES INTO INFANT C3H/ST. AND (C3H/ST. × SWISS)F_1 MICE*

6C3HED cells from	Number of cells inj. intraperitoneally	Into	Average survival time (days)	Percent survivors tumor-free at 105 days
C3H/St. passage No. 134........	200 200	8 C3H/St. 15 F_1	15.6 32.2	0 (0/8) 20 (3/15)
Swiss passage No. 74............	1200 1200	7 C3H/St. 8 F_1	71.2 98.0	29 (2/7) 87 (7/8)

* These four tests were set up simultaneously in December 1953.

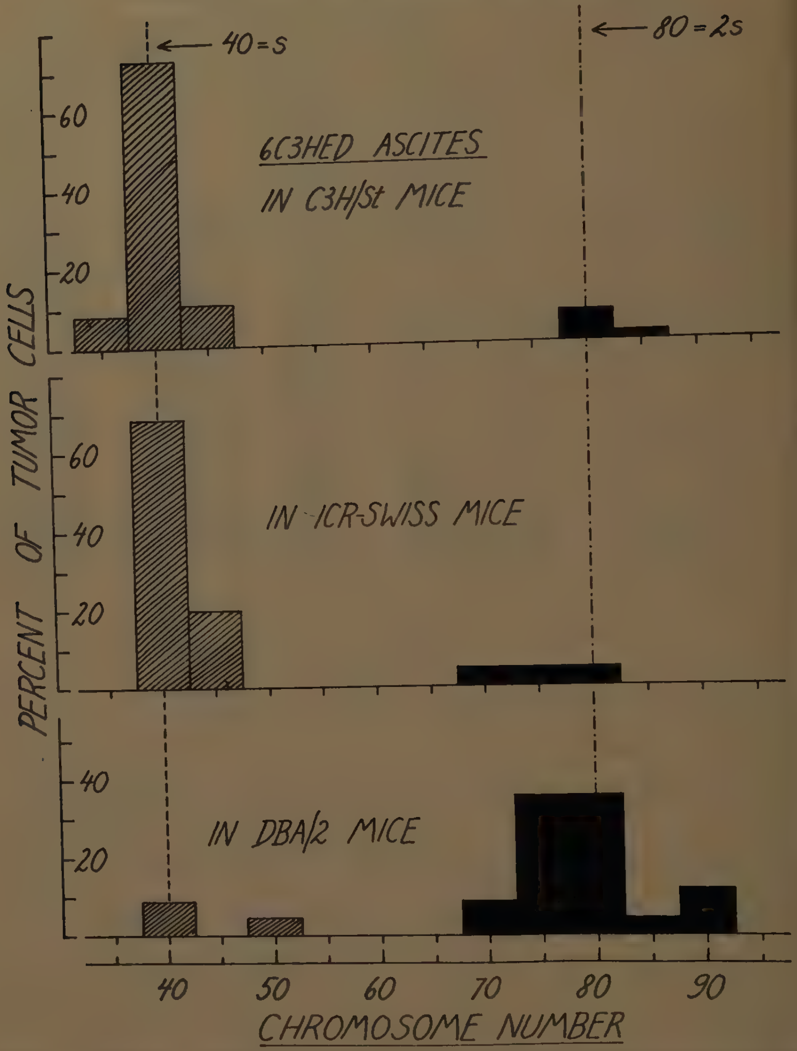

FIGURE 3. Distribution of chromosome numbers in three sublines of the 6C3HED mouse lymphosarcoma.

from its transplantability with blood (Kaziwara, unpublished data). Fiv days after intraperitoneal inoculation of C3H/St. mice, enough 6C3HED cell had reached the peripheral circulation to permit takes in further C3H mic injected with such contaminated blood from the tail-tip (57/57 takes) or fron the retro-orbital sinus (9/9 takes).

(2) *Cytologic comparison of the 6C3HED sublines.* The chromosome consti tution was analyzed periodically in the C3H/St. main line of the tumor, i Swiss, and in DBA/2 serial passage, as well as after return from foreign host to the strain of origin. Percentage of near-diploid and near-tetraploid cell

as determined in aceto-orcein squash preparations made from the ascites uring growth in various genotypes of mice and during successive stages in the istory of the three principal sublines, covering the period of December 1951 to ay 1955. From the photomicrographs of two typical preparations (FIGURE , B, D), it is clear that well-spread near-diploid metaphases (B) are easily istinguishable from doubled chromosome constitutions (D). The proportions f ploidy distribution in FIGURE 2 are based on random samplings of 50 to 00 metaphases each. Details pertaining to the 16 samples in FIGURE 2 are isted in TABLE 4.

There is an obvious relationship between capacity for progressive growth in genetically refractory environment and proportion of heteroploid cells. In he C3H/St. parental strain, the neoplastic cell population retained its largely iploid character over a period of four years. The chromosome constitution n Swiss passage is almost indistinguishable from that in C3H/St. This suggests that the hereditary gap between C3H/St. and Swiss was large enough to revent selection of compatible cells, giving the tumor no opportunity to establish itself in the Swiss stock, even through the genotypic remodeling as-

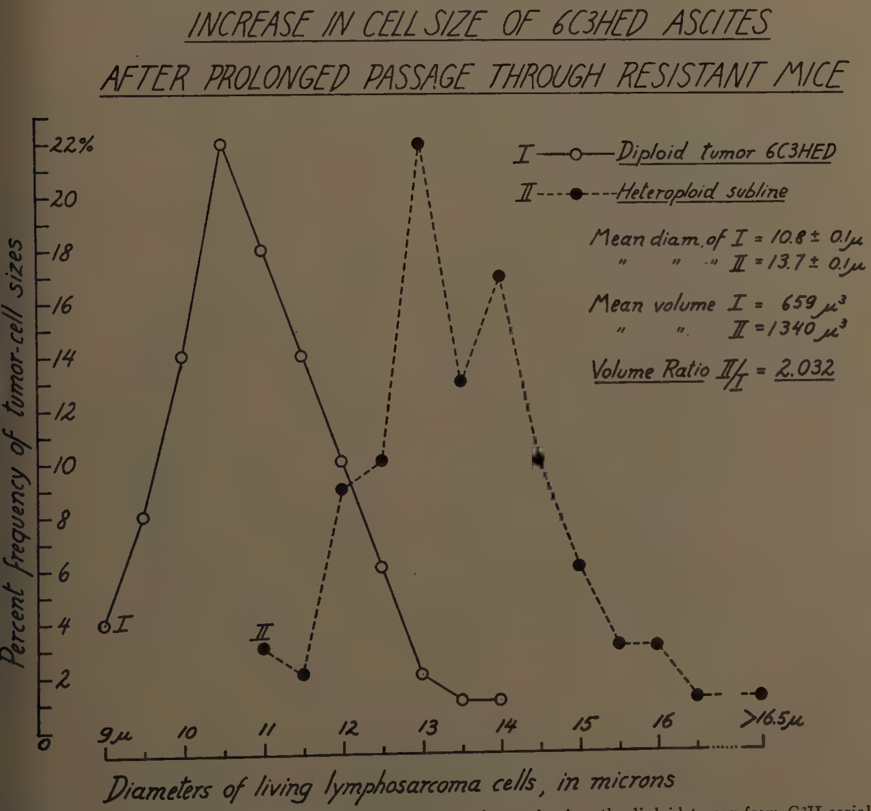

FIGURE 4. Distribution of lengths of cell diameters in the predominantly diploid tumor from C3H serial passage, and its largely tetraploid subline from DBA/2 passage. Ratio of cell volumes is 1:2.

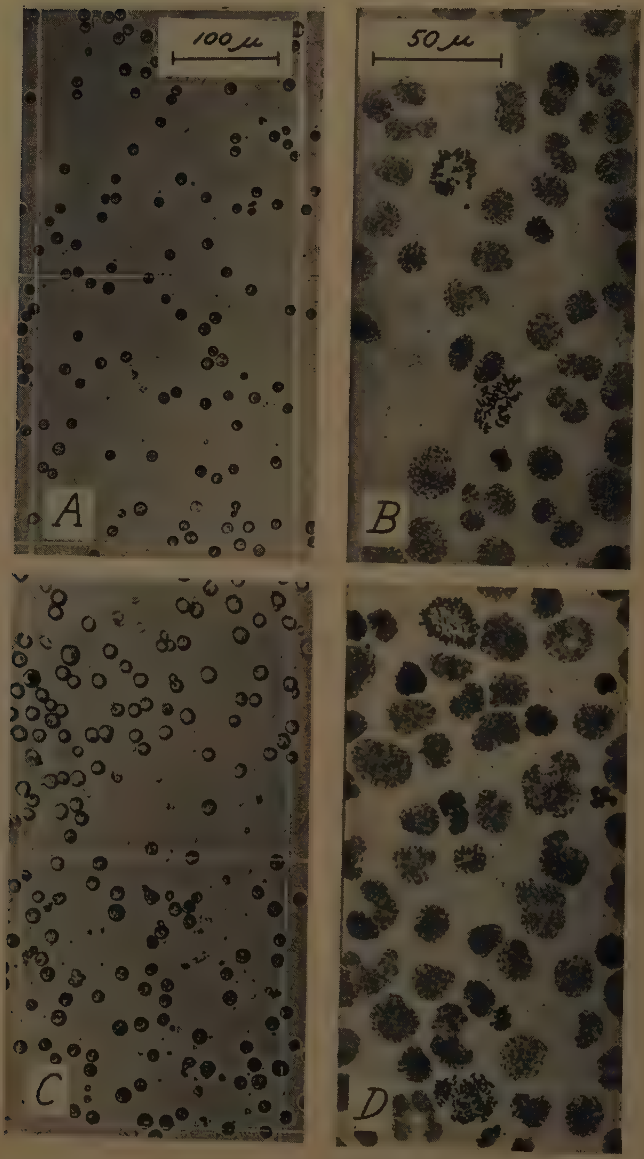

FIGURE 5. Predominantly diploid (s) and near-tetraploid (2s) sublines of the 6C3HED lymphosarcoma ascites.

A. Living cells of the tumor from serial passage in the C3H/St. strain of origin. Ascites diluted 1:100 with mammalian Ringer solution and photographed in an A.O. bright-line counting chamber. The two small squares shown are 250 microns square. Cells are not compressed since the chamber is 100 microns deep.

B. Squash preparation of the diploid 6C3HED tumor line, fixed and stained with aceto-orcein.

C. and D. Corresponding photomicrographs of the near-tetraploid DBA/2 adapted 6C3HED tumor line, photographed at the same magnification as A. and B.

TABLE 4

CHANGES IN CHROMOSOME CONSTITUTION IN VARIOUS SUBLINES OF THE 6C3HED LYMPHOSARCOMA ASCITES DEPENDING ON GENOTYPE OF HOST

Ref. to column in fig. 2	6C3HED tumor subline (Transfer generation No., or special transfer conditions)	Sampling date	Number of metaphases sampled	Chromosome constitution		
				% Diploid ± s	% Tetraploid ± 2s	% Octoploid ± 4s
1	G.28 of serial C3H passage	12/13/51	50	98	2	—
2	G.97 of serial C3H passage	5/15/53	111	91	8	1
3	G.183 of serial C3H passage	11/26/54	100	94	6	—
4	G.202 of serial C3H passage	5/7/55	200	91	7	1
5	G.2 of serial Swiss passage	7/7/52	50	84	14	2
6	G.71 of serial Swiss passage	11/30/53	100	96	4	—
7	G.79 of serial Swiss passage	2/19/54	100	87	13	—
8	Low cell dosage from Swiss G.74 returned to C3H, sampled 120 days later	4/13/54	100	69	31	—
9	*Early* appearing take in (C3H × DBA)*BC* mouse, inoc. from Swiss line	2/14/54	500	98	2	—
10	*Late* appearing take in (C3H × DBA)*BC* mouse, inoc. from Swiss line	4/13/54	100	28	70	2
11	G.2 of serial DBA passage	11/16/54	100	2	91	7
12	G.2a of serial DBA passage	5/3/55	100	6	93	1
13	G.4a of serial DBA passage	4/30/55	300	6	92	2
14	9 days after return from DBA to C3H	5/2/55	100	17	83	—
15	17 days after return from DBA to C3H	5/10/55	110	76	24	—
16	93 days after return from DBA to C3H	2/18/55	100	90	10	—

sociated with heteroploidy. The proportion of viable polyploids may have increased slightly during the last few Swiss transfers, apparent from the 31 per cent polyploid component in a partially immunized C3H mouse represented by column 8, FIGURE 2. In compatible back-cross mice (column 9), the tumor also retained its largely diploid character, but the delayed takes in the more resistant back-cross individuals (column 10) began to exhibit a preponderance of near-tetraploid elements.

The latter tumors made their appearance so late—in some cases three months after inoculation with Swiss 6C3HED—that the possibility of their being spontaneous tumors originating in these BC mice had to be ruled out. The histocompatibility factors segregating in the back-cross were contributed by C3H/St. and DBA/2. A tumor arising in such a back-cross individual (three-quarters DBA/2) should grow best in (C3H × DBA)F_1 mice in which the cellular antigens of both parental types are equally represented. It was shown that the late-appearing tumors in back-cross hybrids were derivatives of 6C3HED, since—after inoculation into C3H/St., F_1, and DBA/2 mice—they were lethal for the former two, but not for the latter.

One out of 32 DBA/2 mice in this test, however, developed a progressively growing ascites that was near-tetraploid and retained a doubled chromosome constitution during successive DBA/2 transfers covering a period of six month (columns 11 to 13). The population shift toward polyploidy went hand i hand with lethal, though very slow, growth in DBA/2 mice which are stil completely resistant to the diploid main line of the tumor.

The heteroploid cells were not selected out because of reproductive advantages over the diploid population component. Actually, they grow at retarded rate, are less invasive, and kill much more slowly than the more virulent diploid lymphoma. Their nonreactivity toward the specific immunogenetic difference between diploid 6C3HED cells and the DBA/2 genotyp (antigen K versus D) is the most probable explanation for their numerica ascendancy This view gains support from the rapid reverse selection towar diploid, after DBA-adapted heteroploid ascites is returned to the more compatible C3H environment (TABLE 4, last three lines; FIGURE 2, columns 14 t 16).

Exact chromosome counts for the lymphoma are made difficult by the crowding of metaphase plates in a relatively small cytoplasm which causes overlapping, especially in the near-tetraploid cells. A chromosome number of abou 90 can nevertheless be worked out for the metaphase shown in FIGURE 6, J. The photomicrographs H and I permit exact numerical determinations of 4 and 42 chromosomes respectively. Additional exact counts for the 6C3HE main line in C3H have been published.[2, 5] Accurate chromosome number for the Swiss-adapted line were obtained for 26 cells as follows: 37, 39, 39, 39 40, 40, 40, 40, 40, 40, 40, 41, 41, 42, 42, 42, 42, 43, 43, 43, 44, 44, 46, 72, 76 80. These values are less subject to slight error from overlapping than th following numbers for 28 metaphases from the DBA/2-adapted line: 40, 40 40, 41, 50, 71, 71, 72, 72, 73, 75, 75, 76, 76, 77, 77, 79, 79, 80, 80, 80, 80, 82 83, 85, 89, 90, 91. A graphic representation of numerical distribution o chromosome numbers in the three sublines, centered on intervals of five, i given in FIGURE 3.

The question arises whether the predominance of near-tetraploid metaphase n the DBA/2 line results from temporary mitotic inhibition of diploid cells or whether the metaphase values adequately represent the cellular composition of the entire neoplasm. Comparative measurements of cell diameters i living, noncompressed cells from C3H/St. and DBA/2 passage are plotted i FIGURE 4. There is little overlap between the two populations, and their cel volumes differ by a ratio of 2:1. This may be seen in the photomicrograph of corresponding cells taken at low magnification in a counting chambe (FIGURE 5, A and C).

Although the DBA-adapted line has become largely polyploid, it still retain the characteristic nuclear lobation and multinucleation that has been described earlier[5] for the diploid 6C3HED. The lobes are apparent in severa of the resting nuclei of FIGURE 5, B and D, in a prophase (FIGURE 6, G), an a giant nucleus (FIGURE 6, L).

A late tetrapolar anaphase containing laggards (FIGURE 6, K) features tw cytologic mechanisms whereby unbalanced heteroploid nuclei with a labil transplantation potential can be accidentally produced. Most of the resulting

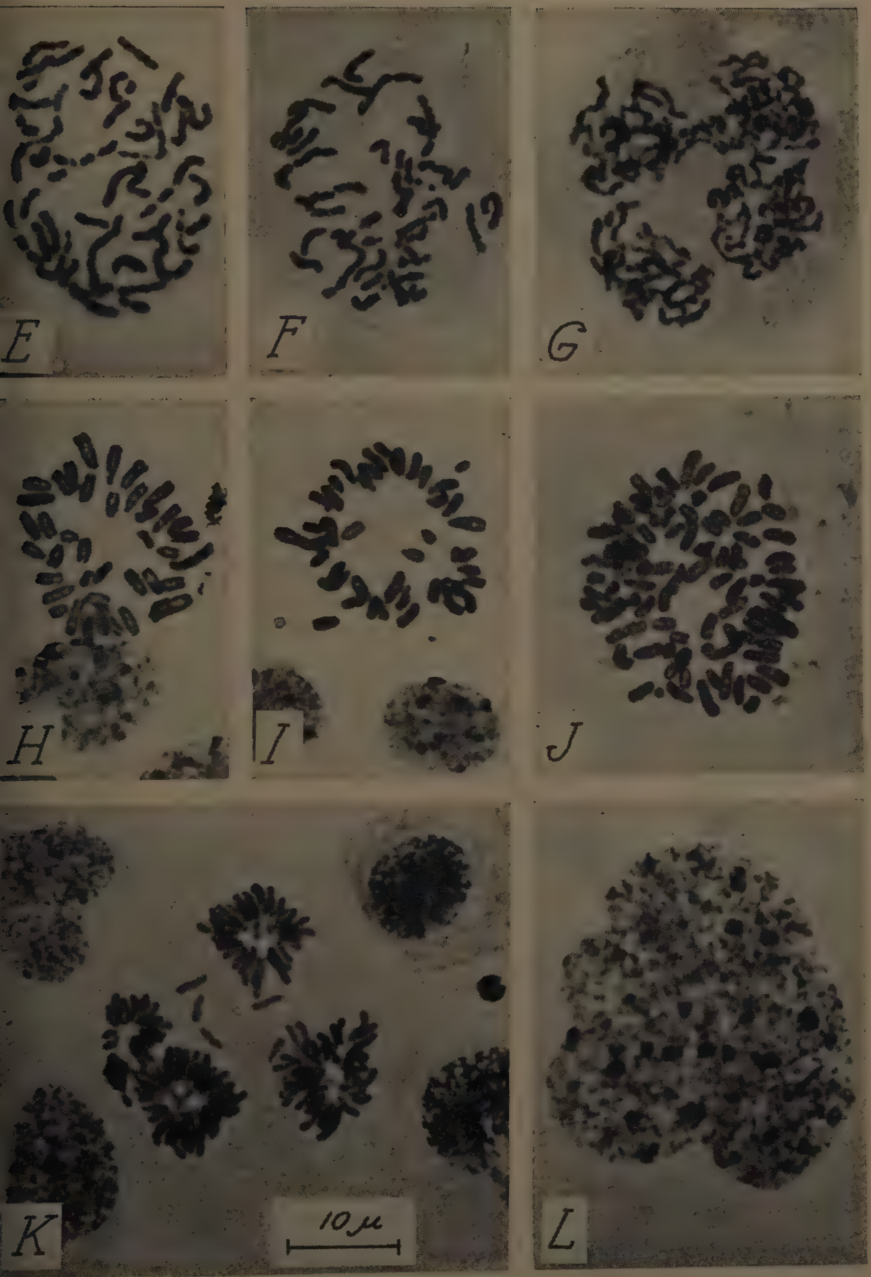

FIGURE 6. Photomicrographs of chromosome conditions in the three sublines of 6C3HED lymphosarcoma.
E. and H. Near-diploid prophase and metaphase from the ascites in serial Swiss mouse passage.
F. and I. Near-diploid prophase and metaphase from tumor growing in C3H mice.
G. and J. Lobated heteroploid prophase and near-tetraploid metaphase from the DBA/2 adapted ascites.
K. Giant polyploid nucleus from DBA passage.
L. Tetrapolar anaphase, occurring rarely in all sublines shows one of several mechanisms for aberration from the balanced diploid genotype. All photomicrographs taken under Köhler illumination at the same magnification with 2 mm. Spencer 90 × 1.3 n.a. apochromatic oil immersion objective, Spencer 1.3 n.a. achromatic condenser (used immersed) and first surface mirror. A Bausch & Lomb research lamp with ribbon filament and Wratten filters No. 58 and No. 15, and a Bausch & Lomb Ampliplan 6× ocular were employed.

TABLE 5

CHANGED ANTIGENCITY OF THE 6C3HED LYMPHOSARCOMA (C3H-ORIGIN) TOWARD DBA/ MICE AFTER SERIAL PASSAGE THROUGH VARIOUS GENOTYPES

6C3HED asictes from	Genotype of test mice	Number of mice inj.	Inj. site	Per cent lethal takes	Number of antigenic H-factors	Mean survival (days)	Moda ploid
C3H/St. passage	(C3H × DBA) × DBA back-cross	331	i.p.	53.2	1	12.7 ± 0.2	s
		328	s.c.	40.9	1 (?)	26.4 ± 1.3	
(C3H × DBA) F₁ passage	(C3H × DBA) × DBA back-cross	192	i.p.	50.0	1	14.3 ± 0.4	s
Swiss passage	(C3H × DBA) × DBA back-cross	66	i.p.	28.8	2	100.6 ± 7.1	s
		68	s.c.	13.2	3	107.9 ± 8.3	
DBA/2 passage	DBA/2	82	i.p.	<100	0	188.2	2s

aberrant cells may not be viable, but the rare exceptions could have immunologic advantages in a refractory host genotype.

(3) *Histocompatibility tests.* The isoantigenic properties of the 6C3HED ascites and its sublines were tested in relation to resistant DBA/2 mice whic differ at the *H-2* histocompatibility locus[12] from the tumor's home strain C3H/St. is homozygous for $H\text{-}2^k$, DBA/2 for $H\text{-}2^d$. Through the work o Gorer and his associates[15, 18, 19, 20] the complexity of the *H-2* locus and rar crossing over between some of its subcomponents have become apparent C3H/St. cells are characterized by the *H-2* constitution *CdEfK* (lower cas letters denoting absence of substances D and F), while DBA/2 cells produc the allelic or pseudoallelic antigens CDE^dF, lacking *K*.

Take percentages in back-cross hybrids of the type (C3H/St. × DBA/2 F₁ × DBA/2 provided information on the number of segregating factors tha invariably cause the diploid C3H lymphoma to regress in refractory DBA/ mice (TABLE 5, line 1).

The main line of the 6C3HED ascites from unbroken C3H passage gav 53.2 per cent lethal takes in the back-cross animals after intraperitoneal injection of 150 million malignant lymphocytes—an excellent agreement wit the 50 per cent monofactorial expectation. Goodness of fit (TABLE 6, line 1 supports the conclusion that the diploid ascites and the DBA/2 stock diffe by one immunogenetic entity which segregates as a single unit, presumably a the *H-2* locus.

Additional tests with the ascites tumor from C3H/St. passage were aimed a comparing its intraperitoneal and subcutaneous transplantability in backcross hybrids (TABLE 5, line 2). The 40.9 per cent lethal subcutaneous take were fewer than expected, fitting neither a one-gene (50 per cent) nor a two-gene (25 per cent) ratio (TABLE 6, line 2). The discrepancy between the tw implantation sites may result from prolonged selection of the 6C3HED cell for growth competence in peritoneal exudate, a medium physiologically distinct from the subcutaneous environment.[21, 22] Reduced subpanicular o intramuscular (by comparison with intraperitoneal) compatibility of ascite tumor grafts, however, is not a general phenomenon, since it was not found i

TABLE 6

LETHAL TAKES OF 6C3HED LYMPHOSARCOMA IN (C3H × DBA)BC MICE

Goodness of Fit for Mendelian Segregation of Isoantigenic Differences

6C3HED ascites from	Test site	Number BC mice tested	Actual lethal takes	Mendelian expectation of takes	Dev.	S.E.$_r$	$\frac{\text{Dev.}}{\text{S.E.}_r}$	p
3H/St. passage	i.p.	331	176	165.5 (1 gene)	10.5	9.1	1.1	>0.26
	s.c.	328	134	164.0 (1 gene)	30.0	9.1	3.3	<0.003
wiss passage	i.p.	66	19	16.5 (2 genes)	2.5	3.5	0.7	>0.45
	s.c.	68	9	8.5 (3 genes)	0.5	2.7	0.2	>0.90

imilar extensive experiments with two A-strain lymphomas (Hauschka and vedar, unpublished data).

The genetic specificity of the diploid ascites was not modified by side passage hrough first generation hybrids between C3H/St. and DBA/2. After the umor had grown temporarily in these susceptible $H\text{-}2^d/H\text{-}2^k$ mice, it was reested in back-cross individuals, where it continued to give 50 per cent takes TABLE 5, line 3). The "induced adaptation" of histocompatibility that Barett and Deringer have obtained by brief F_1 passage,[23] and that has been found o proceed either toward decreased or increased antigenicity in various neolasms,[24, 25, 26] probably cannot supplant a one-gene *H-2* requirement with mmunogenetic indifference.

While F_1 passage could not alter the established Mendelian gap between the umor and DBA/2, prolonged serial transfer in the totally unrelated ICR Swiss mice of the genotype $H\text{-}2^s/H\text{-}2^s$* did increase the graft's genetic differntial with regard to DBA/2 as follows: the Swiss-adapted ascites required two genes for intraperitoneal take in the back-cross and three genes for subcutaneous ake (TABLE 5, lines 4 and 5; TABLE 6, lines 3 and 4).

It is in harmony with our working hypothesis that the malignant lymphocytes were still diploid after this measurable increase in their specificity. One could speculate that a "mutation" in a tumor cell at a histocompatibility locus other than *H-2* agreed with the Swiss genotype, and therefore was selected, while the original *H-2* disagreement between 6C3HED and DBA/2 persisted during Swiss passage. When the tumor was finally retested in (C3H/St. × DBA/2)F_1 × DBA/2 back-cross mice, the antigenic mutant had become abundant because of its better compatibility with Swiss and manifested a new DBA-incompatibility.

Immunoselection with an opposite trend—that is to say, toward decreased specificity—occurred in the DBA-adapted line where the tumor became near-tetraploid after more than two year's sojourn in refractory hosts. This tetraploid subline, judged by its progressive growth in DBA/2 mice, seems to lack the functional antigenic difference between the original diploid 6C3HED and DBA/2.

(4) *Serological differences between near-diploid and near-tetraploid 6C3HED*

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* According to unpublished data by Hoecker and a more detailed analysis by Amos, the *H-2* antigens in ICR Swiss are CEFS. D, K, B are lacking.

TABLE 7

ABSORPTION OF ANTI-K,E BY VARIOUS CELL-POPULATIONS OF 6C3HED LYMPHOSARCOMA AS TESTED BY RED CELL ISOAGGLUTINATION

Material used for absorption	Serial transplant generation	Predominant cell type	Antigen activit
C3H/St. liver (control)	—	Balanced**	8
Tumor from C3H/St.	108 (1953)	Near diploid	5
Tumor from C3H/St.	202 (1955)	Near-diploid	5
Tumor from DBA/2	4 (1955)	Near-tetraploid	1
DBA/2 adapted tumor after return to C3H/St.	1 (1955)	75% diploid	3

* Fall in titer of antibody expressed as number of tubes.

** Polyploid nuclei are arrested in "antephase" and are probably balanced polyploids (J. T. Wilson, pe sonal communication.)

ascites cells. The relationship between chromosomal balance and immun genetic constitution indicated above and in earlier publications[3, 4, 6] woul have to remain speculative in the absence of serologic evidence. The latt was provided through the hemagglutination tests in TABLE 7, which typifi results obtained for over 40 individual absorptions. More detailed data a incorporated in the following paper.[27]

Known anti *H-2* sera directed against antigens K and E were absorbed wit C3H liver, with near-diploid ascites tumor cells from uninterrupted C3 passage, and with near-tetraploid material from the DBA/2-adapted line, an then compared with unabsorbed serum for hemagglutinin titer. Fall in th titer correlates with loss of transplantation specificity and with ploidy change In the last column of TABLE 7, antigenic activity is expressed as loss of hemagglutinating potency by comparison with the unabsorbed control, which averaged 1/4000. "Activity 1" signifies a one-tube drop to serum dilution 1/200 "Activity 5" means removal of antibody down to a titer of 1/128.

These serological results support the conclusion that the diploid lymphom cells, which give lethal takes only in *H-2*^{k} mice, carry antigens E and K. Th tetraploid DBA/2-adapted ascites has lost its E and K activity, at least fro the cell-surface and probably altogether.

A confirmatory increase in the absorptive affinity from activity 1 to 3 wa observed after return of the tetraploid tumor line from DBA/2 passage t compatible C3H/St. hosts, where reverse selection toward diploidy occurre within 17 to 93 days (FIGURE 2).

Discussion

Among the "progressive" changes in cancer tissue, the antigenic fluctuation detectable by transplantation techniques are of paramount interest because:

(1) "Antigenic simplification" has been proposed as a characteristic o neoplasms since they generally have fewer histocompatibility requirements tha nonmalignant homografts.[20]

(2) Loss of certain tissue antigens, by which organ growth is supposedl held within bounds, has been implicated (in theory) as a primary step in th etiology of invasive cancer.

(3) Mendelian analysis of "mutations" in somatic cells is limited to measrable cell functions controlled by known loci in the inbred animals from which he tissue has been derived. The only mouse genes in this category are the istocompatibility factors, especially the *H-2* complex, located in linkage roup IX.[12] Homograft reactions controlled by this locus can be studied in xact genetic and serologic terms.

(4) The causal sequence: heteroploidy → reduced antigenicity → wider ransplantability introduces the problem of genic interaction in the synthesis f isoantigens, although such antigens normally segregate as monofactorial raits.

Changed chromosome numbers, making some cells in a neoplasm less antigenic, would also endow these cells with greater resistance to the isoantibody elicited by the more specific diploid components of the graft. Heteroploid elements thereby attain positive selection value in a genetically refractory animal. The effectiveness of immunoselection as an experimental tool for concentrating the less antigenic cells in a mixed neoplastic population has given additional support to the interrelationship between heteroploidy and reduced specificity.[3, 4]

The ploidy shift from 45 to 90 chromosomes, induced by Kaziwara[10] in the Ehrlich carcinoma after passage through partially immunized Swiss mice, presupposes an immunogenetic mechanism. The hyperdiploid Ehrlich ascites tumor studied by him, however, is not strain-specific, its *H-2* constitution is unknown, and serologic absorption tests were not performed. These disadvantages were eliminated in the present experiments with the 6C3HED lymphoma. The isoimmune gap across which the polyploid cells were selected was defined both in genetic and serologic terms. The cytologic evidence rests on chromosome samplings over a four-year period and includes the original diploid main line of 6C3HED, a diploid derivative with increased genetic specificity (differing from DBA/2 by two genes instead of one), and the near-tetraploid subline capable of growing across the $H\text{-}2^d$ barrier.

Not only homoplastic transplants, but heteroplastic ones as well, may respond to their temporary hosts through alterations in chromosome number. Yosida[28] has described such changes for the Yoshida rat sarcoma during its growth in mice. A significant numerical drop from 80 toward decidedly hypotetraploid modes was observed in heterografts of the Ehrlich mouse carcinoma ascites in hamster passage (Oestergren and Levan, 1955; personal communication).

The readiness with which the 6C3HED lymphoma responds to immunoselection with two opposite trends—either toward reduced or increased specificity—should be remembered in assessing effective immunization[29, 30, 31] of C3H mice against their "own" 6C3HED tumor. During its past history, this tumor was propagated in various sublines of the C3H strain and was inadvertently selected both for and against minor incompatibilities by which the sublines may have differed. Immunogenetic identity is doubtful between the tumor and those C3H stocks that can produce protective antibody against the tumor.

The loss of specificity of the DBA-adapted subline of 6C3HED and th simultaneous ascent to supremacy of tetraploid cells in this line can be inte preted as *immunoselection* for several reasons:

(1) The growth rate of the polyploid cells gives them no competitive ad vantage over the diploids.

(2) The tetraploid line is less virulent and less invasive than the diplo cells.

(3) Hematocrit values for cell-mass in the two lines are comparable per un volume of ascites fluid (unpublished data).

(4) When the tetraploid DBA branch of the tumor is returned to C3 passage, the diploid residue emerges from hiding and again takes over th population.

(5) Thus, the only obvious selective advantage of the polyploid cells in th refractory DBA/2 environment is an immunological advantage, namely th serologically proved weakening of isoantigens.

Reverse selection from the polyploid state toward diploid, after return t the C3H home-strain, has its counterpart in Kaziwara's experiments with th Ehrlich tumor.[10] His polyploid EL88 sublines were at first rather stable, bu they reverted to hyperdiploid when they were transferred through nonim munized mice. They could be forced to become polyploid once more by th simple expedient of low-dosage transfer, which allowed time for the develop ment of effective antibody titers.

It would seem that immunoselection is restricted to specific experimenta situations, where the gene gap between graft and recipient is not so great as t cause irreversible damage to the homograft before a favored cell type gain growth momentum. In ICR Swiss mice, for instance, the genetic obstacle were insurmountable.

After passage through refractory genotypes, various tumors have bee shown to lose some of their virulence upon return to the strain of origin.[32, 33,] This decline in virulence manifested itself as a decrease in take percentage i the home strain and was found to be a "function of length of residence in th foreign strain." Hoecker *et al.* described reduction of takes after a side passag of only seven days. In Mitchison's experiments,[33] the critical growth perio in foreign soil necessary to damage 6C3HED grafts irreversibly was about 1 days. This may explain why our Swiss-adapted line of 6C3HED failed in it 84th passage. Up to passage 74, our transfer interval had been 7 or 8 days For the last 10 serial transfers, it was increased to 9 or 10 days, with cumula tive damage resulting in the extinction of the subline.

With our DBA-adapted line of 6C3HED, now 39 months removed from C3H passage, growth rate after return to C3H is greatly retarded, but letha takes in the strain of origin are still nearly 100 per cent. The reselection from polyploidy toward diploidy in the source-genotype suggests that the original specific diploid cell-population component has persisted all this time in a re tarded latent state.

Sachs and Gallily[35] decided against the possibility of selective advantag among heteroploid elements after too brief an observation period of 6C3HE n Swiss passage. It is impossible to detect a significant population shift luring a single passage, *i.e.*, 8 days. Prolonged exposure to immunologic ressure in various refractory genotypes seems to be a prerequisite for extracting and concentrating antigenic variants which are able to give rise to a new onspecific subline. That this may be a slow process is borne out by the resent experiments.

Also, it is not surprising that a heterologous rat environment, which offends mouse tumor in antigenic as well as in many metabolic features, fails to roduce a noticeable increase of heteroploids within the first week of trial.[35]

In their report confirming earlier observations[3, 4] on the interrelationship etween chromosome number and graft specificity, Sachs and Gallily agree vith our statement that "a simple duplication of the diploid chromosome set s probably not the essential factor in the loss of antigenicity; for if this were so, the rare tetraploids should occasionally survive and give rise to a new cell-population after inoculation into a foreign strain." They are correct in pointing out that chromosome number *range* is less important in the loss or weakening of antigens than *modal* number, to which they assign the term "*fundamental number*." For our somewhat simpler hypothesis, which ascribes the loss of immunogenetic specificity to chromosomal imbalance, they substitute the concept of "*the right duplicate chromosomal material* for the establishment of nonspecificity .... It may, in fact, be possible to determine which and how many chromosomes must be *duplicated* for the establishment of various degrees of strain specificity."

This emphasis on an intrinsic role or duplication is contradicted by several facts. Duplication, triplication, and omissions of chromosomes, as well as translocation have been seen. All these changes would result in genic imbalance, and sometimes in the physical loss of an antigenic locus. About half of the chromosome number modes in our surveys of 17 mouse ascites tumors and clones derived from them are uneven numbers between 41 to 47 and 71 to 79. Similarly, Sato[36] found that 44 of 100 exactly counted metaphases in the relatively nonspecific Yoshida sarcoma had uneven numbers. Unequivocal counting is preferable, as a basis for discussion, to grouping chromosome numbers in multiples of five. Judging from Yerganian's[37] precise idiogram analyses in a transplantable sarcoma of the Chinese hamster, triplication or higher multiples, as well as the "absence of all representatives of a specific chromosome type in viable cells" can occur.

The "fundamental numbers" found by Sachs and Gallily in their ascites material are thought to represent "different degrees of duplication of 40, the normal diploid number of the mouse," and it is stated that these numbers are "very close to a polyploid series of 40, 60, 80." Chromosome numbers around 60, however, are quite rare in our experience, and they mimic the triploid number only superficially. There is no mitotic mechanism that would permit such erratic tumor cells to acquire constitutions genetically equivalent to a triploid idiogram.

The most essential feature of heteroploidy, in so far as it affects gene-controlled antigenicity of cells, is genic imbalance. This is not necessarily a handicap, but it could be accompanied by certain growth advantages. Addition of a single chromosome to a balanced diploid idiogram may alter the cellular antigens as drastically as doubling of the entire set, followed by subtraction of a few chromosomes. Chromatin loss is probably frequent, because modal numbers between 70 and 78 are the most common among the polyploid mouse tumors, none of which is rigidly strain-specific.

The early observations of Strong,[38] Bittner,[39] and others on stepwise losses of transplantation requirements as well as changes from a lower to a higher gene-determined specificity[24, 25, 32, 40] can be interpreted as somatic "mutations." There is no disagreement between this view and the findings of Hauschka and Levan,[3] Sachs and Gallily,[35] and Amos[27] regarding the decline of specificity when tumors become heteroploid. Heteroploidy appears to be causally connected only with the final step from a graft-host difference involving one or more genes to the disappearance of an effective immune barrier. This final step, of course, could, come about also through point mutation. But the present evidence implicates gross chromosome number changes and the ensuing drastic upheaval of the total genotype as the most likely causes of genetic indifference in tumors.

H-2 characteristics seem to persist longer than antigenic properties determined by other histocompatibility factors and cannot easily be obliterated as long as genic balance remains intact. At *H-2* and other *H*-loci of diploid cells, one antigenic condition may mutate to an allelic character. The possibility of true antigenic mutation in diploid tumor cells is reinforced by the discovery of such mutations in the diploid germinal tissues of mice. C57 Black, for example, has changed from $H\text{-}2^b$ to $H\text{-}2^d$ (Borges and Kvedar).[41] There is, as yet, no recorded case of actual loss of an *H-2* property in a diploid tumor.

This tenacity of *H-2* suggests that the locus plays an essential role in the life of the cell beyond merely controlling antigenicity. Perhaps it participates with other genes in shaping the reactive configuration of the cell membrane.[27]

Gorer[20] attributes evolutionary significance to the polymorphism at the *H-2* locus. Although "antigen II" (the original name for the products controlled by *H-2*) is not very mutable in pure stocks, mice as a species are rather heterogeneous at this locus. It is assumed that the histocompatibility alleles perform functions beyond their part in homograft immunity.

Need the *H-2* antigens be completely eliminated to permit progressive growth of a tumor in an alien strain, or are they diminished in amount below a protective threshold?

Gorer's concept of antigenic displacement[42] can be brought into hypothetical accord with the reduced specificity of heteroploid cells. Strong antigens, effectively distributed over the surfaces of the ancestral diploid type, are perhaps "crowded out" by inactive substitutes, after accidental shifts in chromosome number have upset synthetic equilibria.

The ratio of cell volume to surface may be important in determining how much functional antigen reaches the surface. Measurements of cell diameter for the two sublines of 6C3HED (FIGURE 4) have shown that the mean volume of the tetraploid cells is twice that of the corresponding diploids. The cell surface, however, is only 59 per cent greater. This could upset the correct proportion of antigen to antibody necessary to inhibit the polyploid cells.

An alternative to the "crowding out" of *H-2* antigens is the possibility that they are products of a principal gene subject to the influence of many minor modifiers.

The regularity with which haphazard drift in chromosome number and loss of functional antigens have coincided would imply that the differentiation of strain-specific antigenic end products requires a reaction series sensitive, at successive stages of synthesis, to all sorts of interferences arising from dosage changes inherent in heteroploidy. Normally nonparticipating genes, thus, may exert indirect effects on the formation of antigens that, in a balanced diploid cell, behave as direct, rather than mediate, products of the determining loci.

A third genetical interpretation suggested by interaction between alleles at the *Rh* locus in man cannot be disregarded. Ceppellini, Dunn, and Turri[43] have shown that a *Cde* chromosome paired to a normal *D* depresses reactivity sometimes to a point resembling the much weaker response of typical D^u bloods. D^u phenotypes, thus, are either the direct product of the D^u allele or phenocopies resulting from coincidence of *Cde* with *D*. Interactions of this kind may be common in transplantable neoplasms, as aneuploidy creates functional rearrangements comparable with heterozygosis in formerly almost identical chromosome homologues ("identical," because derived from sib-mated strains).

Since the glyco-lipo-protein lattice of the cell surface is probably controlled by numerous genes, any change in the total dosage relationship of loci to one another would tend to displace, alter, or inactivate molecules with specific antigenic groupings located on the cell membrane. It is primarily through the cell surface that the host recognizes or rejects the graft.

The hemagglutination tests of Amos[27] have provided the most crucial evidence for the diminution of certain *H-2* antigens as a consequence of heteroploidy in the 6C3HED ascites, and the reappearance of these same antigens after reverse selection toward diploidy. Confirming Hoecker's unpublished data for two A-strain lymphomas (1954), Amos investigated two additional pairs of diploid and heteroploid ascites tumors. In these comparisons, the polyploid cells were far weaker absorbers of isoantibody than the diploids.

Mutually supportive cytologic, genetic, and serologic criteria, therefore, justify interpretation of the wider homotransplantability of heteroploid cancer cells as immunologic selection of the least antigenic types from among the viable chromosomal variants in the neoplastic cell population.

Summary

The role of immunoselection in the relationship between heteroploidy and reduced specificity of tumor homografts was investigated. Three sublines of the 6C3HED lymphosarcoma ascites (C3H origin) were compared in cytologic, genetic, and serologic respects:

The principal tumor line is highly virulent and invasive, has a diploid chromosome number mode, is genetically compatible only with $H\text{-}2^{k}$ mice, differs from DBA/2 by one histocompatibility factor at the *H-2* locus, and is characterized by antigens CEK.

A near-diploid subline, transferred through 84 serial passages in the Swiss ($H\text{-}2^{s}$) strain, has always regressed in these mice, has lost much of its former virulence, but retained its compatibility for C3H. During its long indirect transfer through refractory mice, the requirements for lethal growth in DBA/2-BC hybrids have increased from one to two independently segregating genes.

The DBA/2-adapted subline has a near-tetraploid chromosome constitution and is mildly virulent and noninvasive, requiring more than six months to kill DBA/2 mice. The isoantigenic specificity of these cells is reduced to feeble hemagglutinin absorbing titers by comparison with the potent diploid 6C3HED material.

A small residue of diploid lymphoma cells, giving an $H\text{-}2^{k}$ reaction, persists in the tetraploid ascites This minority is gradually reselected toward predominance in the neoplastic population after the tumor has been returned to the C3H home-strain.

Discussion centers on mechanisms whereby heteroploidy may affect the differentiation of isoantigens.

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SEROLOGICAL DIFFERENCES BETWEEN COMPARABLE DIPLOID AND TETRAPLOID LINES OF THREE MOUSE ASCITES TUMORS

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The apparent relationship between chromosome constitution and genetic specificity described in the preceding paper[2] and elsewhere[3] calls for critical immunologic study of comparable diploid and tetraploid tumor pairs.

Three such pairs in the ascites form were available for investigation: the lymphosarcoma 6C3HED and its polyploid variant; the two A lymphomas No. 1 and No. 2; and two branches of the A-strain carcinoma TA3.

Serologic differences have been studied in two series of experiments. In the first, a number of known anti H-2 sera were tested for their hemagglutinin titer before and after absorption with tumor cells, using the method described by Gorer and Mikulska.[1] In the second, antibodies prepared against the tumors in various noncompatible inbred strains of mice were titrated in the same manner.

Absorption of various anti H-2 stock sera. The results of a typical experiment with the A lymphomas are shown in TABLE 1. The antibody Balb-C anti A-strain sarcoma MTC 1 (H-2D anti H-2A) agglutinates A-strain red cells to a titer of 8,000, C3H cells to a titer of 4,000, and reacts very feebly with C57 Bl cells. After a single absorption with the diploid A No. 2 ascites cells, all hemagglutinating activity is lost at the dilutions tested, but after absorption with tetraploid A No. 1 ascites cells, the titers remain practically unchanged (this agrees with unpublished data of Hoecker). With some less potent sera, there is some absorption of anti D, but the antigenic difference between the two lymphomas remains clear.

TABLE 2 gives a comparison between the diploid ascites lymphosarcoma 6C3HED grown consistently in the C3H strain and its polyploid DBA/2-adapted subline taken from DBA/2 mice. The results shown under the heading "6C3HED from C3H" are typical of the hemagglutinin titers after absorption with a range of tumor cell samples from the 7th transplant generation frozen in the tumor bank in 1951, up to the 203rd transfer in May 1955. The DBA-adapted cells have all been taken from the current generations 3 and 4, over a period of several months. Up to 6 ml. of ascites fluid can be removed from the much distended DBA mice without causing distress.

The diploid form of the C3H tumor is capable of absorbing anti K and anti E (anti C is not tested for at the dilutions shown), but less efficiently than C3H liver. This is more pronounced with the higher titer antibodies. The diploid cells do not affect the titer for anti D. The DBA adapted polyploid C3H tumor appears quite inert with these sera, the titers after absorption remaining virtually the same as in the control.

TABLE 3 shows that a similar relationship exists between the two TA3 tu-

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TABLE 1

ABSORPTION OF AN ISOIMMUNE SERUM OF THE TYPE H-2D ANTI H-2A BY ASCITES CELLS FROM TWO A-STRAIN LYMPHOMAS

R.B.C. from mouse strain	Controls (No. antiserum)		Titration tube No. and dilution of antiserum								Antibody
	C1	C2	1 128	2 256	3 512	4 1024	5 2048	6 4000	7 8000	8 16000	
A/Ha	—	—	+++	+++	+++	+++	++±	++±	++	±	BALB C anti MTCI (anti K, E) unabsorbed
C3H/St	—	—	+++	+++	+++	+++	++±	++	±	—	
C57B1	—	—	+	±	—	—	—	—	—	—	
A/Ha			—	—	—	—	—	—	—	—	Absorbed with A No. 2 lymphoma cells (near diploid)
C3H/St			—	—	—	—	—	—	—	—	
C57B1			—	—	—	—	—	—	—	—	
A/Ha			+++	+++	+++	++±	++±	++	+	tr	Absorbed with A No. 1 lymphoma cells (near tetraploid)
C3H/St			+++	+++	+++	++±	+±	+	—	—	
C57B1			±	tr	—	—	—	—	—	—	

TABLE 2

THE ABSORPTIVE AFFINITIES OF DIPLOID AND TETRAPLOID CELLS OF 6C3HED FOR ANTI-C, D, K. AND ANTI C, K. OR E, K. ISOIMMUNE SERA

(Hemagglutinin titers expressed as reciprocals)

Red cells of genotype	Unabsorbed (control)	Antisera absorbed with		
		C3H liver (control)	6C3HED from C3H (diploid)	6C3HED adapted to DBA/2 (tetraploid)
		A. Anti C, D, K		
H-2K	256–2048	0	0–128	1024–2048
H-2D	512–1024	512–1024	512–1024	512–1024
		B. Anti K, E or anti C, K		
H-2A	4000–8000	0–64	64–512	4000–8000
H-2K	4000	0	128	2048

mors. The diploid form is consistently highly antigenic, while the aneuploid form is much less active. There appears to have been a little absorptive activity against anti F, but anti C, D, and E appear almost unaffected. It is interesting to note that there is some absorption of anti B by the diploid TA3. This cross reaction is frequently found and is explained by the finding of Gorer (personal communication) that B is an allele of D. TA3 is much less active in this respect where D, if present, is poorly represented.

Antibodies produced against the two A-lymphomas and the two forms of 6C3HED. Ascites cells from each of the A-lymphomas (H-2A) were injected into mice of resistant strains and the immune sera were tested against mouse

Table 3

Comparison Between the Absorptive Capacities of TA3 and TA32 for Various Isoimmune Antibodies

(Hemagglutinin titers expressed as reciprocals)

Red cell of genotype	Type of antibody	Effective antibody	Unabsorbed (control)	Absorbed with TA3 (diploid)	Absorbed with TA32 (aneuploid)
H-2A	H-2D anti H-2A	anti K, E.	8,000	64	4–8,000
H-2Q	H-2D anti H-2A	anti E.	1,024	0	512
H-2A	H-2B anti H-2A	anti C, D, K.	4,000	0	2,000
H-2Q	H-2B anti H-2A	anti C.	1,024	32	1,024
H-2A	H-2K anti H-2B	anti F.	1,024	0	128
H-2B	H-2K anti H-2B	anti B, F	2,048	512	4,000*
H-2A	H-2K anti H-2A	anti D, F.	2,000	0	1,024
H-2Q	H-2K anti H-2A	anti F.	64	0	16
H-2A	H-2B anti H-2K	anti C, K.	1,024	0	1,024
H-2Q	H-2B anti H-2K	anti C.	64	0	64

* Control 8,000.

Table 4

Comparison Between Antibodies Produced in C57 Black Against A-Strain Diploid Lymphoma No. 2 and Near Tetraploid Lymphoma No. 1

(Red cell titers expressed as reciprocal)

Red cells of genotype	Unabsorbed	After a single absorption with				
		A liver	L No. 2 (Diploid)	L No. 1 (Tetraploid)	DBA/2 liver	C$_3$H liver
Antibody—C57 Bl anti L No. 1						
H-2A	1,024	32	32	64	64	512
H-2K	16	0	0	0	0	0
H-2D	256	16	0	16	0	256
Antibody—C57 Bl anti L No. 2						
H-2A	4,000	—	32	2,048	1,024	2,048
H-2K	1,024	—	0	512	512	0
H-2D	4,000	—	32	4,000	128	4,000

red cells of various known H-2 genotypes. Some of the results with the sera obtained from C57 Bl (H-2B) are shown in TABLE 4. A similar dosage scheme was adopted in preparing both antisera. The antibody against A No. 2 reacted to a titer of 4,000 with A and DBA/2 cells and to 1,024 with C3H cells. It was almost completely cleared by lymphoma No. 2 but was unaffected by lymphoma No. 1, while DBA/2 liver reduced the titer for DBA/2 cells and to some extent for A cells. C3H liver removed the antibodies against C3H, leaving DBA/2 unaffected. These results indicate that the antibody against lymphoma No. 2 contains a strong anti D and anti K and behaves in the same manner as other A-strain tumors previously studied.

TABLE 5

COMPARISON BETWEEN ANTIBODIES PRODUCED IN C57 BLACK AGAINST DIPLOID 6C3HED CELLS FROM C3H MICE AND TETRAPLOID 6C3HED CELLS FROM DBA/2 MICE

(Titers expressed as reciprocals)

Red cells of genotype	Antibody C57Bl antidiploid 6C3HED from C3H		Antibody C57Bl antitetraploid 6C3HED from DBA/2	
	Unabsorbed	Absorbed with DBA/2 liver	Unabsorbed	Absorbed with DBA/2 liver
H-2A	4,000	4,000	512	16
H-2Q	32	8	16	—
H-2D	32	0	64	0
H-2K	8,000	4,000	32	16

The data for antibody against A lymphoma No. 1 are in sharp contrast with this. The initial titers were lower, especially for C3H, which was agglutinated to a titer of only 64. The antibody was easily absorbed by either of the lymphomas or by DBA/2 liver, whereas C3H liver removed only the agglutinins against itself. This antibody appears to be a weak anti D with a very weak anti K and possibly anti C component.

A similar experiment with antibodies against the two C3H lymphomas is summarized in TABLE 5. The antibody against the diploid tumor is a very good anti K. It appears to include some anti C, which reacts with the H-2Q and H-2D cells. The anti C is removed by absorption with DBA/2 liver, while the anti K is unaffected. This is the reaction that would be given by a typical antibody of the type H-2B anti H-2K.

The antibody produced against the tetraploid form of the 6C3HED tumor from DBA/2 is weak, reacting with A cells to a titer of only 512, and reacting somewhat more strongly with DBA/2 cells than with C3H. Its activity is almost completely abolished by a single absorption with DBA/2 liver and it appears to be a weak anti D, with some anti C and, possibly, a very feeble anti K.

A number of absorptions have been performed with the antibody produced against the diploid cells, and these appear to show considerable differences from absorptions made with Gorer's stock antisera. This inconsistency is under investigation, since there may be a difference between the anti K produced against other A and C3H tumors and that produced against 6C3HED.

The general impression from the serological findings to date is that they give close support to Hauschka's theory of the correlation between alterations in chromosome number (genic balance), strain specificity, and antigenicity. The foregoing experiments show that the diploid tumors are more active than the polyploid sublines not only in absorbing antibody from a number of immune sera, but also in provoking an immune response. The fact that polyploid tumors are not entirely indiscriminate in their histocompatibility requirements (*e. g.* L No. 1, 6C3HED in DBA) may be due in part to metabolic differences between graft and host. This point can be decided by attempting passive transfer of immunity to the polyploid cells, as has been done with the immunity to the C57 Bl leukosis EL4.

The antibodies resulting from the injection of large quantities of polyploid cells of A No. 1 lymphoma and 6C3HED may be due to the presence of normal host lymphocytes in the ascites fluid. Differential counts for the former showed approximately one fifth of the cell population to be nonmalignant; 16 per cent lymphocytes and polymorphs of normal diploid size, <3 per cent small histiocytes, <0.2 per cent large macrophages, and 81 per cent tetraploid malignant lymphocytes.[4]

The cytologic and transplantation data of Hauschka and Levan[3] suggest that loss of antigenicity is a general attribute of polyploid tumors. The three pairs of closely related tumors examined above are in good agreement with this hypothesis but, owing to the complexity of the factors governing the fate of a homograft, a larger number of tumors must be examined serologically before the correlation between ploidy and antigenicity can be accepted as a safe generalization.

The nature of the antigenic change is unknown. While some of the H-2 antigens appear to be absent or present in diminished amount, this could be due simply to a change in the nature of the cell surface. Conversely, the interesting link between gene balance and H-2 specificity (*i. e.* an antigenic surface property of the cell) indicates that products of the H-2 locus may perform functions in cellular economy and growth beyond the somewhat unnatural determination of compatibility between graft and host.

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GROWTH CHARACTERISTICS OF FREE TUMOR CELLS IN VARIOUS BODY FLUIDS AND TISSUES OF THE MOUSE*

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The famous Scotch physician John Abernethy used to open his lecture on troubles of digestion with a sentence: "The stomach is neither a stewing pan nor a test tube, but just the stomach." Similarly, I may outline my approach to the subject under discussion by the brief statement that an ascites tumor is neither a tissue culture *in vivo*, nor a bowl of goldfish. It is just a pathological condition of the peritoneal cavity. Experimental evidence reveals that this condition is an association of two phenomena, etiologically related but essentially different: (1) accumulation in the peritoneal cavity of a serous fluid, due to implantation of tumor cells into the peritoneum, and (2) free tumor cell growth in this fluid due to the transformation of interdependent tumor tissue cells into free tumor cells. Therefore, the ascites in the so-called ascites tumor is the result of tumor cell implantation and the free tumor cell growth is the result of cell transformation. Both conditions are initiated by similar material—a suspension of tumor tissue cells. In many instances, however, these cells are capable of implanting and inducing ascites but are unable to initiate free tumor cell growth. Moreover, free tumor cell growth in ascitic fluid can be transferred only by free tumor cells, while the implantation-eliciting ascites can be transferred serially from mouse to mouse either with free tumor cells or with tumor tissue cells. Briefly, an ascites tumor is an association of transmissible ascites and transmissible growth of free tumor cells. The association of two conditions is obligate only for free tumor cells. Ascites does not imply the occurrence of free tumor-cell growth, but growth of free tumor cells is impossible without ascites. The occurrence of ascitic effusion was given major consideration by early investigators (Hesse, 1927;[1] Loewenthal and Jahn, 1932;[2] Seeger, 1937;[3] Haagen, 1934;[4] and Lettré, 1940[5]), and it inspired the term ascites tumor. This term may obscure the fact that tumor growth and implantation are the primary factors of the phenomena labeled as ascites tumors. Moreover, it may wrongly suggest that the mechanism of ascites formation is independent from tumor cell growth and similar to the pathogenesis of experimental ascites of hepatic origin. Shields Warren and Olive Gates[6] in 1936 were the first to recognize the primary importance of tumor cells in an ascites tumor of Walker's carcinoma in rats, which they named "peritoneal carcinosis." I introduced in 1948 the term "free tumor cell growth in the peritoneal fluid," emphasizing the cellular nature of the phenomenon and the secondary role of the ascites.[7]

This "malignant cell ascites" is produced by leakage of serous fluid from small veins and capillaries in the peritoneal subserosa after infiltration with tumor

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cells (provoking anoxia and low-grade inflammation). This leakage occurs wherever intraperitoneally injected cells and fragments of malignant tissue find suitable anatomic conditions for abundant implantation into peritoneum. The classic paper by Rous and Jones[8] described the importance of slight trauma to serosa for tumor cell penetration into subserosa, and Drinker[9] discussed the general conditions of fluid leakage from capillaries. In the absence of favorable conditions, the cells may not implant into the peritoneum, or may implant without causing ascites.

When numerous cells implant and induce ascites, however, other cells lacking topographic prerequisites for implantation remain on the surface of the serosa. This can be shown by repeated washings of the cavity at various intervals after intraperitoneal injection of tissue cell suspensions. The cells escaping implantation become suspended in the accumulating fluid and are enriched by cells and clumps separating from growing implants. If the cells in suspension are able to multiply rapidly, they initiate the second condition—that of free tumor cell growth. If they grow only slowly, however, they may still obtain their chance to find a suitable environment for implantation by being transferred with the fluid into new mice, where they reproduce the same succession of implantation and formation of effusion containing tumor cells. Such is the mechanism of transferable ascites without free tumor cell growth.

The amount of accumulated fluid in ascites of various etiology is directly proportionate to the extent of leakage from the capillaries and inversely to the rate of fluid removal from the cavity by lymphatics and veins, as was shown by several investigators (see literature cited by Drinker[9] and Cunningham[10]). Recently, Prentice, Siri, and Joiner,[11] at Donner Laboratories in California, and Courtice and Simmonds,[12] at Kanematsu Institute in Australia, have demonstrated the rapid turnover of ascitic fluid and pointed out that "effusions are not sequestered and stagnant pools of fluid."

We were able, with the help of a special technique, to use any tumor strain for initiation of ascites with mashed tissue suspensions and for serial transfers of this ascites from mouse to mouse with the ascitic fluid. These results were achieved by inoculating our material into areas of the peritoneal cavity highly suitable for cell implantation, particularly, into the peritoneum covering the female genital organs (FIGURES 1 to 4) or into the mesentery (FIGURES 5 to 6).

If tumor cells escaping implantation multiply rapidly as free cells in the ascitic fluid, their growth rate increases with each transfer into new mice, as I have shown in 1948 for sarcoma 37, sarcoma 180, and thymoma (malignant lymphoma).[7] This was attributed to serial selection of tumor cells with high growth potential. I described, at the same time, my method for experimental selection of fast-growing cells in the following terms: "Each series was initiated by intraperitoneal injection of sarcoma cells, from mashed subcutaneous S-37 implants, into eight mice. Their peritoneal fluid was withdrawn after 4 days and examined. The specimen showing the highest number of sarcoma cells and of their mitoses was used for the transfer of peritoneal fluid into eight new mice, in doses of 0.1 to 0.25 cubic centimeter. After an interval of 3 days, the peritoneal fluid of these mice was examined, and a suitable specimen was selected for use in the second transfer into a new group of six mice. After the first 15 to 20 transfers of each strain, the peritoneal fluid of most animals contained at each transfer a large number of sarcoma cells, and the selection of the 'best' donor was no longer necessary." Our experience indicated that the ability to be transformed into free cells was inherent to some cells of solid tumor used for initiation of the free growth, and could be interpreted either by their higher growth potential or by their greater potency of adaptation to a fluid medium. The choice of interpretation is irrelevant, as both characteristics are only two facets of the same feature—higher autonomy or higher grade of malignancy. Thus, we were first to outline that high growth potential, as an expression of higher autonomy, is a specific biological characteristic of free tumor cells. Other investigators have found special morphological characteristics of their chromosomes (Hauschka, 1952)[13] and of their cytoplasm (Craigie and Lasnitzki, 1952).[14, 15]

Briefly, tumor tissue cells induce ascitic effusion if they are implanted in a suitable area of the serosa, while their conversion into free growth depends on the degree of their autonomy. Therefore, the formation of ascites is determined by the environment of tumor cells and the free cell growth by their intrinsic growth potency.

So far, the initiation of free tumor cell growth by selective transfers of rapidly growing cells or, in other terms, the conversion of tumor tissue cells into free cells, has failed with some tumor strains. There is no evidence, however, on which to attribute these failures to any intrinsic characteristics of these strains and not to limitations of present routine technique. It should be remembered that, in several instances recorded in the literature (and probably in many unreported cases), some investigators failed to convert tumor tissue into ascites tumor where earlier or later investigators were successful. For these reasons, I should not label any strain as inconvertible and commit myself to a classification of tumor strains with regard to their ability for conversion. It has been my experience that the difference between various mouse tumors with regard to their ability to be converted into free growth cells is only quantitative and largely dependent on the technique of manipulation. I have noted repeatedly that each specimen of mouse tumor is an agglomerate of cells of various degrees of malignancy or autonomy as manifested by their growth potential in ascitic fluid. The problem of tumor tissue conversion into free cells is dealt with in my experiments as a technical problem of increasing the proportion of cells with higher malignancy by experimental selection.

The occurrence of free tumor cell growth in ascitic fluid, as I have repeatedly emphasized,[16] is only a special case of free tumor cell growth in body fluids and tissues. In collaboration with Barbara Jeffries, I have obtained free tumor cell growth in the pleural effusion of the mouse, using S-37, S-180, thymoma, carcinoma H, melanoma, and other strains[16] (FIGURES 9 and 10), and have found with each tumor some special conditions for cell implantation into the pleura (FIGURES 11 to 14). Another example of free tumor cell growth is the presence of mitotic leukemic cells in the blood. In our experiments with three strains of mouse leukemia (AK4, P1534 lymphatic leukemia in DBA-2, and C1498 granulocytic leukemia in C57-6), we have shown[17, 18, 19] that primary growth of leukemic cells as free cells can be initiated in peritoneal or pleural effusions

(FIGURES 7 and 8) and followed by the spread of cells from these effusions into blood and into organs. On the other hand, we have demonstrated that free cells of ascites tumors also invade the blood and the visceral organs—in smaller numbers, however—and with a tendency on the part of infiltrating cells to grow as organized implants. These results show that there is only a quantitative difference in the growth characteristics of free cells as distinguished from leukemic blood and from ascites tumors.

For quantitative estimation of free tumor cell growth in the ascitic fluid, it should not be forgotten that the total amount of this fluid in the peritoneal cavity depends on the balance of fluid leakage and fluid drainage, which are both subject to fluctuation. Moreover, the number of tumor cells in the fluid is decreased continuously or intermittently by their implantation. An ascites tumor should not be compared, for the purpose of computation of its cell number, with a stagnant fishpond enclosed by concrete walls, but rather with a stream where the water level is changing and where fish breeding in the water quite often become stuck in the mud. Several agents affecting permeability of capillaries (cortisone[20]) or inducing their obstruction by hemorrhages [heparin (unpublished observation)] induced marked fluctuations of the ascitic fluid and of its content of tumor cells. What is more, cells may disappear from the fluid under the influence of agents promoting their implantation (by trauma to the serosa, by deposit of fibrin, *etc.*). Finally, some agents inhibit the removal of the ascitic fluid by occluding lymphatics and blood vessels (which may be obliterated also by growing implants) and increase the volume of ascites, thus decreasing cell concentration. Therefore, the amount of ascitic fluid may be accepted as reflecting the level of tumor cell multiplication only in its early stage and in untreated animals, while, in later growth stage and in treated animals, it is subject to variations by other factors.

Concentration of tumor cells in the ascitic fluid increases progressively in the early growth stage and, for some tumor strains, until the death of the host. We were able to reduce, by special technique, individual variations of cell concentration in the ascitic fluid of untreated mice to not more than 10 per cent. The drop of tumor cell number per cubic millimeter, by more than 20 per cent after treatment, indicated direct or indirect effect (fluid changes, increased cell implantation) of the agent on tumor cells. The results of cell counts in the fluid were interpreted in the light of the biological test of tumor cell viability outlined by me in 1950.[21, 22] At various intervals after treatment of a mouse with ascites tumor, a standard dose of ascitic fluid is transferred intraperitoneally from the treated mouse into a new mouse. Lack of intraperitoneal growth in the new mouse is an evidence of irreversible damage to tumor cells in treated animals. This damage is attributed to a direct effect on tumor cells. In our experiments, this effect was closely associated with mass appearance of abnormal mitoses (Haddow, 1949;[23] Koller, 1947[24]). It has been obtained, so far, only with nitrogen mustard or by gamma or beta irradiation.[21, 22, 25, 26, 27, 28]

To repeat in a few words: (1) only in early stages of an ascites tumor and in untreated mice, may the volume of ascitic fluid be considered consistently as a reflection of the amount of tumor cell growth; (2) a drop of tumor cell concentration in the early growth stage is evidence of a direct or indirect effect of some factor or agent on tumor cells; (3) irreversible damage to tumor cell viability, as demonstrated by the failure of cell transfer into new mice, is an indication of a direct effect on free tumor cells.

The tendency of free tumor cells from ascites tumors to spread by blood route into organs as metastatic emboli has been demonstrated in rats by Shields Warren and Olive Gates in 1936[6] and, recently, in mice by Julian and Clara Ambrus.[29] The role of single cells in production of metastases has been outlined by Coman[30] and by Renato Baserga.[31]

For detection of metastatic tumor cell emboli or of their growth in organs, we have introduced the method of organ grafts from tumor-bearing mice into the peritoneal cavity of new mice.[30] It was found that the peritoneal cavity acts as a selective medium for growth even of small numbers of tumor cells nestling in tissues. They grow either as free cells in the exudate or as implants on the serosa, while normal tissue elements are disintegrated and removed by macrophages. We have applied this phenomenon for screening the effects of radioactive and chemical agents on metastatic emboli: if the intraperitoneal growth is obtained by inoculation of an organ from at least 50 per cent of all untreated controls, the failure of organs from treated animals to initiate intraperitoneal growth (perhaps, with a few exceptions only) is considered as evidence of metastatic cell inactivation by the agent.[32]

Using the above method for detection of metastases in the blood and in organs of mice bearing primary tumor growth at various sites of the body, we have found, in animals with primary free tumor cell growth in the peritoneal or pleural cavity, that the blood and the organs contained tumor cells considerably more frequently and abundantly than in animals with subcutaneous solid tumors (FIGURES 15 to 19). Accordingly, free tumor cells are characterized by the increase of both growth and spread potentials.

Why do mice with ascites tumors die much earlier than mice with subcutaneous or intramuscular tumors of the same strain? Why do free tumor cells growing in the ascitic fluid kill their host much faster than large masses of tumor tissue? In a paper due to appear in the next issue of *Cancer Research*, I have endeavored to show that, not the amount of tumor cell growth, but the pattern of tumor cell spread appears to be the essential lethal factor in many instances. Among mice inoculated by various routes with the same number of malignant cells of the same tumor strain, the animal with free tumor cell growth in the pleural exudate died first. It was found at the autopsy that even a few tumor cells infiltrating from the exudate into the lung frequently induced edema and hemorrhages. By a similar mechanism, tumor cells from ascitic fluid abundantly invaded the liver, the pancreas, and, less often, other abdominal organs, inducing atrophic changes and hemorrhages. These effects were scarce and delayed in mice with solid tumors. The difference is attributed to a higher spread potential of free tumor cells from serous fluids. These data may bear on the little explored problem of clinical pathology: Why do human patients die from cancer? But that is another story.

The ability of tumor tissue to be dissolved in a fluid medium into free cells with high growth and spread potential can be attributed to the most essential and specific characteristics of these cells—their autonomy. Tumor cells are usually considered to be only tissue cells. The role of their second type of growth—the free cell growth—in the pathogenesis of malignant disease has not been much investigated. It may be presumed that further study will bring more data concerning the occurrence of free tumor cells in various body fluids and tissues and concerning their role in the pathogenesis of malignant conditions.

In association with Doctor G. J. Tarleton,[23, 24] we have found that free tumor cells are more sensitive to beta and gamma irradiation and to chemical agents (perhaps on account of their higher percentage of mitoses) than tissue cells. These results may suggest the hope that tumor cells spreading in the blood and tissues may provide an easier target for therapeutic approach than solid tumors. This hope is encouraged by outstanding clinical results in the treatment of some free tumor cell conditions and in leukemias and pleural or peritoneal carcinosis (intracavitary treatment).

In this short review, however, I have endeavored to summarize, not my results, but my methods. I have intended to show that in the use of ascites tumors as a tool for quantitative oncology, the primary test object is the free growth of tumor cells in the serous fluid and their spread into tissues by implantation and by lymph and blood routes. Growth of tumor cells and their spread are inseparably associated in malignant conditions. Ascites tumors offer the unique advantage of screening the direct effect of various agents on both components of malignant disease: on tumor cell growth and on tumor cell spread. It is not unreasonable to believe that the field of research on ascites tumors will grow into a much larger field of free tumor cell pathology in various body fluids and tissues of tumor-bearing host, and that the quantitative method initiated by ascites tumor study will gain higher precision by accepting as its object the whole body of the host and not only his abdominal cavity.

Summary and Conclusions

(1) The pathological condition of the peritoneal cavity induced by malignant cells and known as "ascites tumor" is an association of two phenomena: (a) accumulation in the peritoneal cavity of a serous fluid due to implantation of tumor cells into the peritoneum, and (b) free tumor cell growth in this fluid due to the transformation of tumor tissue cells into free tumor cells. Thus, ascites is the phenomenon of tumor cell implantation and the free tumor cell growth—the phenomenon of cell transformation. In many instances, tumor cells capable of implanting and inducing ascites were unable to grow as free cells in the ascitic fluid. The first condition can be transferred serially by ascitic fluid containing either free tumor cells (which were shown by the author to possess higher growth potential than tumor tissue cells) or by cells and fragments from peritoneal implants. The second condition is transferable only by fluid containing free tumor cells.

(2) The volume of ascitic effusion is the result of the excess of serous fluid leakage from peritoneal capillaries over its removal by lymphatics and veins. The leakage is due to implantation (infiltration or organized growth) of tumor cells into the peritoneal subserosa. It occurs only if tumor cells suspended in the fluid find suitable conditions for implantation. Therefore, their random

inoculation into the cavity may fail. The author, however, was able to induce and to transfer ascites with any tumor strain by using a special technique of inoculation into areas highly suitable for implantation (pelvic peritoneum).

(3) Conversion of tumor tissue cells into free cells was obtained by the author in 1948–1949 with several tumor strains (S-37, S-180, thymoma, *etc.*) by selective serial transfers of tumor cells with highest growth potential. Failure of conversion of some tumor strains was attributed to imperfections of routine techniques and not to any intrinsic incapacity of tumor strains.

(4) Ascites tumors are only a special case of a general phenomenon, that of free tumor cell growth in body fluids such as pleural exudate (Goldie and Jeffries, 1951) and leukemic blood, and in tissues. Metastatic cell emboli were detected (Goldie *et al.*, 1953) by the method of organ grafts from tumor-bearing mice into new mice. In mice with ascites tumors or free tumor cell growth in the pleural exudate, the blood and the organs were found to contain tumor cells more frequently and abundantly than in mice with solid tumors. Thus, free tumor cells are characterized by the increase of both growth and spread potentials.

(5) "Ascitic effusion is not a stagnant pool" (F. C. Courtice and W. J. Simmonds). It has a rapid turnover (T. C. Prentice, W. Siri, and E. E. Joiner) and it can be considerably decreased by agents affecting blood capillaries, or increased by occlusion of lymph and blood vessels responsible for fluid drainage. Therefore, the volume of ascites may be considered as a reflection of tumor cell growth only in its early stage and only in untreated mice. The concentration (number per cubic centimeter) of tumor cells in the fluid reflects their growth also only in its early stage, both in untreated and treated animals. Its drop, after treatment of the host, indicated indirect (reversible) or direct (irreversible) effect on tumor cell viability. The latter is recognized by biological test of cell viability (Goldie *et al.*, 1950), *i.e.* by intraperitoneal transfer of ascitic fluid from treated into new mice. Failure of growth is an indication of the irreversible (direct) effect on tumor cells.

(6) Detection of metastatic tumor cells by organ grafts (Goldie *et al.*, 1953) provided a tool for screening the effect of agents on metastatic tumor emboli. The higher sensitivity of free tumor cells to irradiation (Goldie and Tarleton) inspires the hope that tumor cell spread in the blood and tissues may prove to be an easier target for treatment than growth of organized tumors.

(7) It is concluded that, in ascites tumors, growth of free malignant cells and their spread are inseparably associated. Both are suitable as test objects for investigating, on quantitative lines, the mechanism of malignant conditions and the effect of various agents on malignant cells.

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PLATE 1

FIGURE 1. Melanoma S-91 growth in the peritoneal cavity 30 days after intrapelvic inoculation. Large amount of ascitic fluid. Large lateral tumor masses separated by the intestine which carries a very small nodule. Numerous very small nodules on the peritoneal serosa.

FIGURE 2. Same mouse. Peritoneum opened, fluid drained, intestine shifted aside. Tumor masses in the pelvis, nodules scattered over the peritoneal serosa, including its diaphragmatic insertion.

FIGURE 3. Carcinoma E0771 growth in the peritoneal cavity 12 days after intrapelvic inoculation. Peritoneum opened, fluid drained. Uterine body and horns thickened, tumor masses symmetrically arranged on both sides of the uterus and on the ovaries.

FIGURE 4. Sarcoma 180 intraperitoneal growth seven days after intrapelvic inoculation. Note resemblance to growth shown in FIGURE 3.

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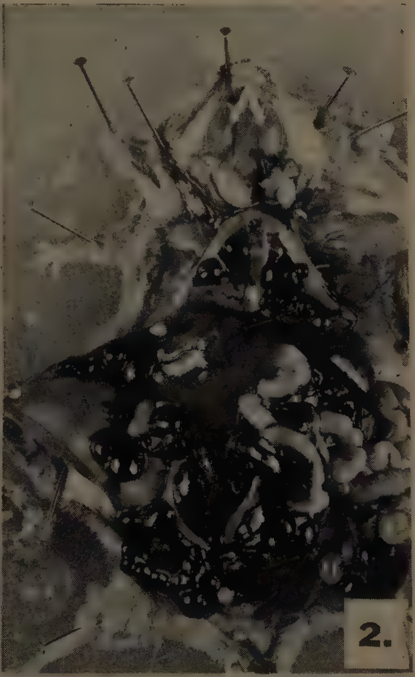
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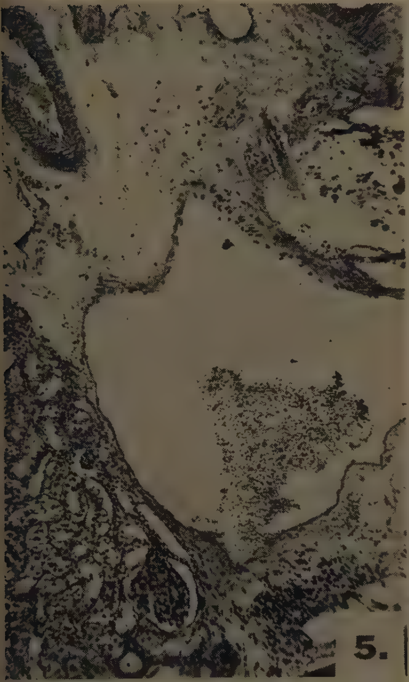
PLATE 2

Figure 5. Intraperitoneal growth of sarcoma 180 six days after inoculation. Infiltration of perirenal fa tissue. Hematoxylin-eosin. ×50.

Figure 6. Mesentery, four days after intraperitoneal inoculation of a massive dose of Sarcoma 37 cells. Massive infiltration, largely perivascular, of fat tissue.

Figure 7. Free tumor cells in the ascitic fluid of the mouse on the fifth day after intraperitoneal inoculatio of 10^6 leukemic cells. AK4 leukemia. Wright. ×600. Practically pure culture of leukemic cells.

Figure 8. Same, P-1534 leukemia.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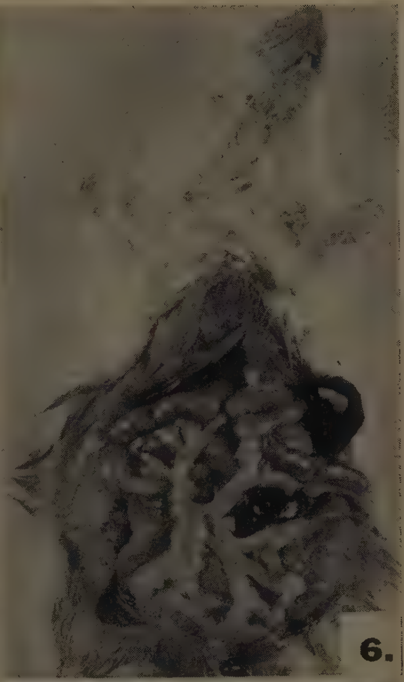
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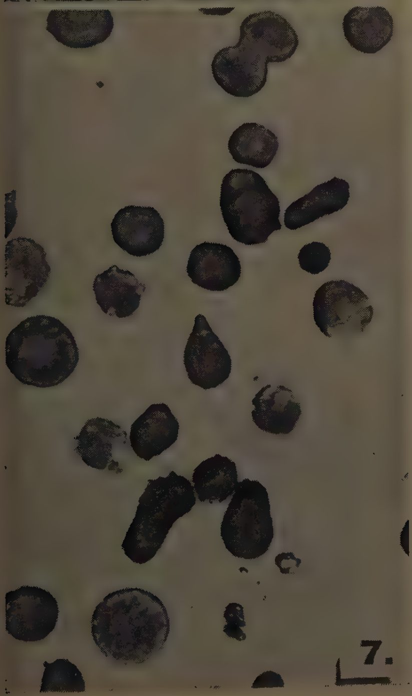
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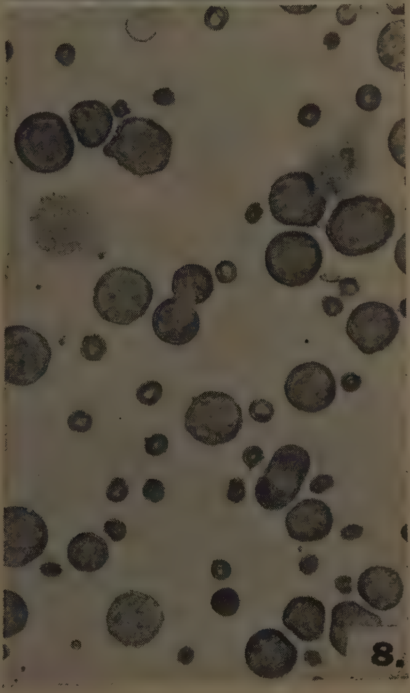
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PLATE 3

FIGURES 9-14. Free tumor cell growth in the pleural exudate and their implantation in the pleura. (Reprinted from: Cancer Research. 1952. **6:** 422–425.)

FIGURE 9. Carcinoma (C3H mice) free cells in the pleural exudate 12 days after inoculation. Aceto-orcein ×600.

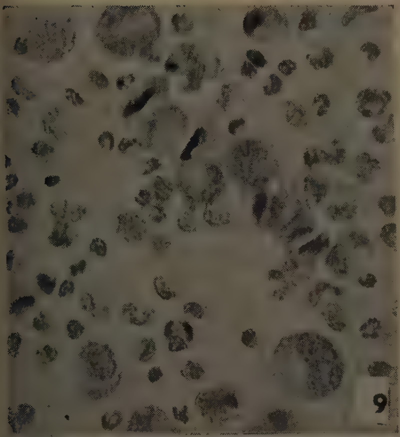
FIGURE 10. Melanoma, five days, Aceto-orcein, ×600.

FIGURE 11. Sarcoma 180 implant in the pleura, nine days.

FIGURE 12. Sarcoma 37, 12 days.

FIGURE 13. Carcinoma in C3H, 15 days.

FIGURE 14. Melanoma, 45 days.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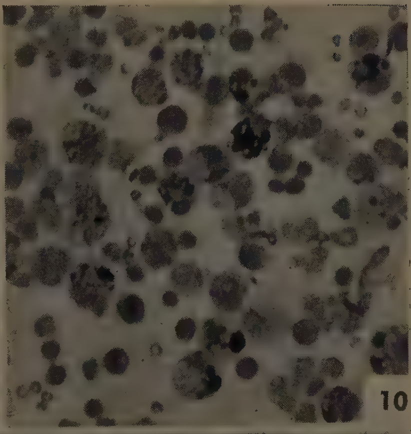
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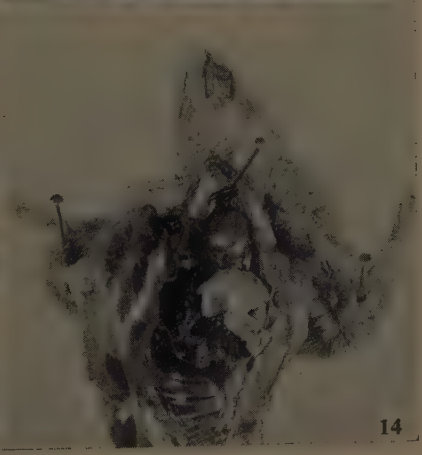
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PLATE 4

Spread of free tumor cells in organs. Hematoxylin-eosin. FIGURES 15–17 reprinted from: Cancer Research 1955. **15**: 263–267.

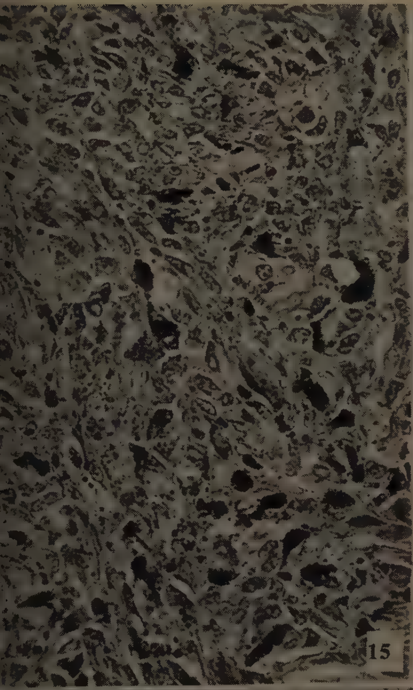
FIGURE 15. Liver, 40 days after intraperitoneal inoculation with melanoma (Harding-Passey) cells, invasion of liver tissue by scattered melanoma cells. ×200.

FIGURE 16. Lung, four days after intrapleural inoculation with sarcoma 180 cells. Tumor cell infiltratio from the pleural exudate. ×175.

FIGURE 17. Liver, 10 days after intraperitoneal inoculation with carcinoma E0771 cells. Small groups c free cells in sinusoids. ×100.

FIGURE 18. Pancreas, six days after intraperitoneal inoculation with S-180 cells. Neoplastic invasion. ×10

FIGURE 19. Brain, 12 days after inoculation of ascitic fluid containing numerous S-180 cells into the scalp Infiltration of brain tissue with tumor cells inducing hemorrhage. ×200.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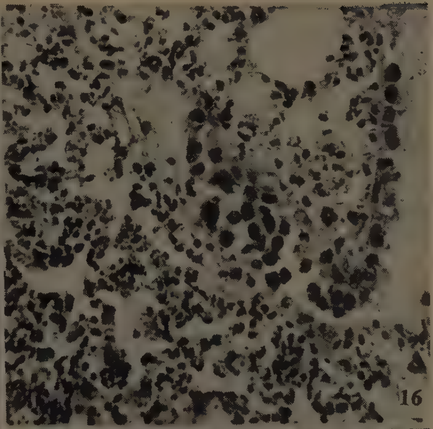
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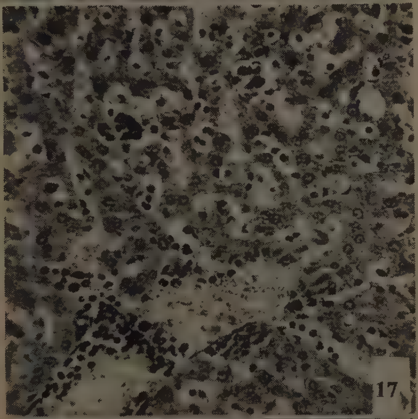
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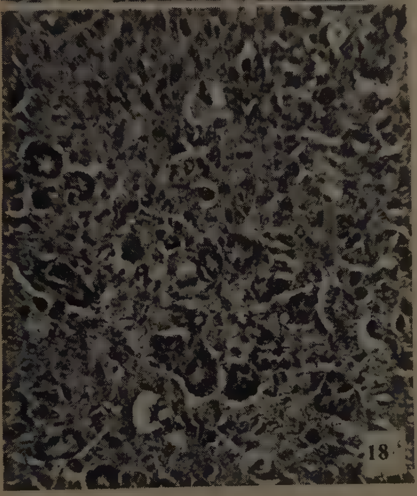
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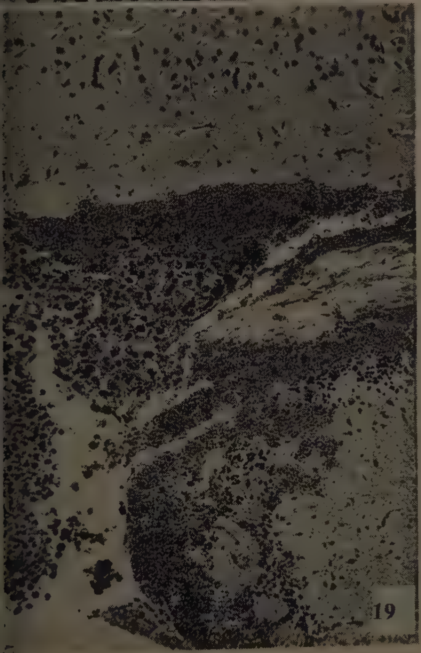
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MEASUREMENT AND NATURE OF ASCITES TUMOR GROWTH

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One might perhaps anticipate a pattern of growth for ascites tumors that conforms to the growth of populations generally. This is so because most ascites tumors consist initially of a suspension of mainly neoplastic cells growing in the peritoneal cavity. Although the over-all situation is more complex than, for example, in the growth of microorganisms in a closed system, there is, in fact, a common thread in the general course of cell multiplication. The capacity for individual growth under certain conditions, which distinguishes the ascites from the solid tumor, suggests a number of intriguing biochemical and physiological problems in addition to other more obvious applications. This paper will be concerned with the nature of ascites tumor growth and methods for its characterization.

Measurement of Ascites Tumor Growth

Evaluation of the growth pattern would appear to be less complicated for ascites than for solid tumors, since the problem may be approached directly by enumeration of the total number of free tumor cells in the peritoneal cavity. This supposition is based on the assumptions that the free cells represent the large majority of all tumor cells in the host, and that the ascites is a homogeneous suspension of predominantly tumor cells. It is generally believed that infiltrative growth is mainly a secondary manifestation of ascites tumor development, its magnitude at any given time depending upon the size of the inoculum and the type of tumor. Under optimal conditions, the initial phase of growth in the free form is probably sufficiently rapid to preclude any serious contribution by solid foci. The number of cells removed from the effective ascitic pool by adherence or organized growth on peritoneal surfaces increases with time and with the magnitude of the ascites.[1, 2] Since invasive growth occurs earlier with ascitic lymphomas than with the Ehrlich or Krebs tumors, the period during which free cells reflect over-all tumor growth would be correspondingly shorter for the lymphomas.

Although solid growth is a variable complicating factor, particularly in the later stages of tumor development,[1-4] it is noteworthy that the volume of ascites may be related linearly to the number of free tumor cells during the first days of growth.[5-7] It is of interest also that there appears to be a direct correlation between the number of free tumor cells of the Ehrlich ascites and the accumulation of plasma proteins in the peritoneal cavity, and that an effect of this sort can be demonstrated within 30 minutes after inoculation.[8]

The population of free tumor cells may be measured by attempting a quantitative recovery of the peritoneal contents by draining and rinsing at sacrifice,[6] or by dye or radioisotope dilution technique in conjunction with cell counts. Evans blue,[5] bromosulfalein,[3] and labeled protein[9] have been used in the latter instance for determination of ascitic volume which, with cell counts nd packed cell volume, permits calculation of the total number of free cells. t may be noted that for some ascites tumors there is a close correspondence etween tumor cells estimated from stained preparations and large cells numerated in a hemocytometer.[5] Hence, large cell counts may be used for many routine purposes in place of differential analyses of stained material.

A variant of the dilution technique in which a given number of labeled tumor ells is injected intraperitoneally just prior to sampling is also applicable.[10] umor cells may be labeled with acriflavin for this purpose. Representative elds on air-dried smears are photographed, using conventional optics and isible and ultraviolet light, the latter to evoke fluorescence in the labeled ells. The percentage of fluorescing cells is determined by projection of the ilms on a grid, the total number of free tumor cells being calculated from he labeled-cell dilution. Tumor cells have been enumerated also by deermination of the DNA content of aliquots of peritoneal fluid.[11] In this nstance, the total number of tumor cells is estimated from the differential ount of ascitic fluid by assuming average DNA values for the various cell ypes.

Comparison of the results obtained with these methods in several mouse scites tumors reveals, in general, a close correspondence. A common limitaion is the appreciable error with small numbers of cells, *i.e.*, less than 25 to 0 × 10^6. This is a reflection, in part, of sampling in addition to other operaional errors. With ascitic volumes in excess of 1 ml., which corresponds to bout 100 × 10^6 Krebs or Ehrlich tumor cells, the error of the dye-dilution echnique is about 5 per cent.[5, 6] The dye and cell dilution methods would eem to be advantageous for study of individual animals. The draining and insing procedure, on the other hand, is particularly applicable for single determinations on pooled samples. It should be pointed out that analyses can be made at leisure with the acriflavin cell dilution method, thus permitting the ampling of large numbers of animals at any one time. The DNA method may be appropriate for ascites tumors that contain an appreciable number of cell aggregates in addition to free cells.

Characteristics of the Growth Response

In general, ascites tumor development consists of an initial phase of rapid cell multiplication followed by a period of decelerated growth toward an asymptote. Although the entire growth pattern has not been characterized mathematically, it appears to be a function mainly of the total number of free tumor cells. The over-all growth picture more nearly resembles a logistic type of function and is in many respects comparable to the growth of microorganisms.

Initial phase. It is not entirely clear whether there is a significant latent period or lag in tumor development immediately after inoculation. Observations with several mouse ascites tumors are somewhat conflicting. A decrease or constancy in cell number during the first day or two has been reported for the Ehrlich, MC1M, and S3A ascites tumors.[6, 12] In contrast, the results of other studies with the Krebs-2 ascites tumor[7] suggest that there is little if any detectable delay in growth onset with inocula of 10^6 to 20 × 10^6 cells. This conclusion is based on the finding that the intercepts of lines derived during the early phase of growth are in essential agreement with the initi inoculum values. Data obtained by others for the Ehrlich ascites[13] and sever ascitic lymphomas[3] support this conclusion. The presence or absence of a appreciable lag in tumor development may, of course, be a consequence innate differences in the growth rates of the various tumors, as well as a re flection of differences in the percentage of actively growing tumor cells in th various inocula. It is pertinent to recall that sampling and other errors measurement are considerable during the first hours after tumor inoculatio Hence the validity of observations based on few animals is open to some ques tion. For this reason, we are inclined to place greater reliance on the indirec criterion cited above, which suggests that a latent period, if it exists, must b comparatively brief, at least for some of the mouse ascites tumors with inocul in excess of 10^6 cells.

Growth curves have been reported for the Ehrlich, Krebs-2, MC1M, S3A 6C3HED, DBA, and EL4 ascites tumors of mice. In all cases, tumor cel multiply rapidly for a variable period and then increase somewhat more slowl to a maximum. According to Patt and Blackford,[7] growth of the Krebs- ascitic carcinoma is essentially exponential over the range from 10^6 to 2×10 tumor cells. An exponential increase in the number of cells during the firs few days of active growth has also been observed by Lucké and Berwick[13] an by Straube *et al.*[10] for the Ehrlich ascitic carcinoma, by E. Klein[12] for the S3 ascitic carcinoma, and by Révész and Klein[3] for several ascitic lymphoma Klein and Révész,[6] on the other hand, have expressed the view that there is n true exponential phase with the Ehrlich and MC1M ascites tumors but rathe a progressive increase in the mean generation time from the onset of growt They believe that a major part of the growth curves of these tumors may b expressed linearly in terms of the cube roots of the number of tumor cells. I is suggested that this formulation may permit comparison with the growth solid tumors. The validity of such comparison, however, is open to question. In agreement with these findings, it may be noted that a cube root transforma tion is also applicable to the intermediate portion of the growth curve of th Krebs-2 ascites tumor.[7] There is an initial exponential phase in the latte instance, however, that extends over a greater range of cell numbers than th cube root transformation. It is well to recall that early exponential growt has also been observed independently for the Ehrlich as well as for othe ascites tumors.

Intermediate and terminal phases. The initial phase of active growth is fol lowed in all instances by a period of progressively declining growth. It is interest that leveling becomes apparent when a certain maximum number tumor cells has been reached. The asymptote may differ somewhat for th various tumors (TABLE 1). That growth may be limited to a maximal tumo protoplasmic mass is shown by comparison of the growth curves of the diploi Ehrlich ascites tumor and a tetraploid subline of the same tumor. Accordin to Hauschka and Grinnell,[14] the diploid cell volume is about half of the tetra ploid. Although growth rates are comparable, the final cell number of th former is approximately twice that of the latter. The leveling of various asciti lymphomas begins at about the same cell number despite a difference in cel

TABLE 1

GROWTH CHARACTERISTICS OF SEVERAL ASCITES TUMORS

Ascites tumor	Mouse strain	Cell volume u^3	Inoculum, 10^6 cells	Doubling time, hours	Appr. asymptote 10^6 cells	Reference
hrlich	Swiss C57BL	2188	20	21	1200	13
"	C3H/St	1903	1.8	12–78	1500	6
"	C3H/St	1903	17.5	13–52	1500	6
rebs-2	CF1	2000	20	16	1000	7
3A	A/St	1508	1	12*	800	12
C3HED	C3H/St	719	20	9	800	3
"	C3H/St	539	80	10.5	800	3
BA	DBA/2	585	20	7.6	1200	3
L4	C57BL	393	20	5.2	1200	3
"	C57BL	397	80	10.1	1200	3

* Estimated from curves.

olume.[3] This picture, however, is complicated by their early metastatic rowth.

The leveling phase no doubt reflects a complex situation to which many hysical and chemical factors may contribute. We must think, for example, n terms of the increasing competition among cells for the various nutrients nd the diminishing capacity of the host to supply them, the accumulation of oxic metabolites, the loss of cells from the ascitic pool by adherence and organized growth, and the progressive deterioration of the host. It is of interest hat liver regeneration and ascites tumor growth are mutually depressed in artially hepatectomized tumor-bearing mice.[15] It may be recalled that the percentage of eosin-resistant and presumably viable cells is relatively constant luring tumor development, but that the mitotic index is decreased during the nore terminal period.[5] There is some evidence that the number of free tumor ells actually may decline during the last few days of life. Indications of a egative exponential growth terminally are reported for the Ehrlich tumor,[13] ut this is not a characteristic finding.

There is reason to believe that the ascites tumor growth pattern is a function nainly of the total number of free tumor cells. The duration of the initial apid growth is related to the size of the inoculum, at least over a certain range. Decelerated growth for a particular tumor becomes obvious in each instance vith a comparable number of free tumor cells. This is clearly evident in FIGURES 1 and 2, where the data for several inoculum groups of the Krebs-2 scites tumor have been transposed to a common time axis.[7] It would appear hat the growth rate of the Krebs ascites does not vary appreciably with increase in the inoculum dose from 10^6 to 20×10^6 cells. There is some indication, however, that the growth rate of the Ehrlich tumor may decrease with arge inocula, *e.g.*, 40×10^6 cells.[6] It may be noted from these figures that ell multiplication is more nearly exponential when the number of tumor cells s comparatively small. With increasing cell numbers, the growth rate gradually leclines, and it is this phase to which a cube root transformation is partially pplicable. An intermediate area may be approximated with equal accuracy

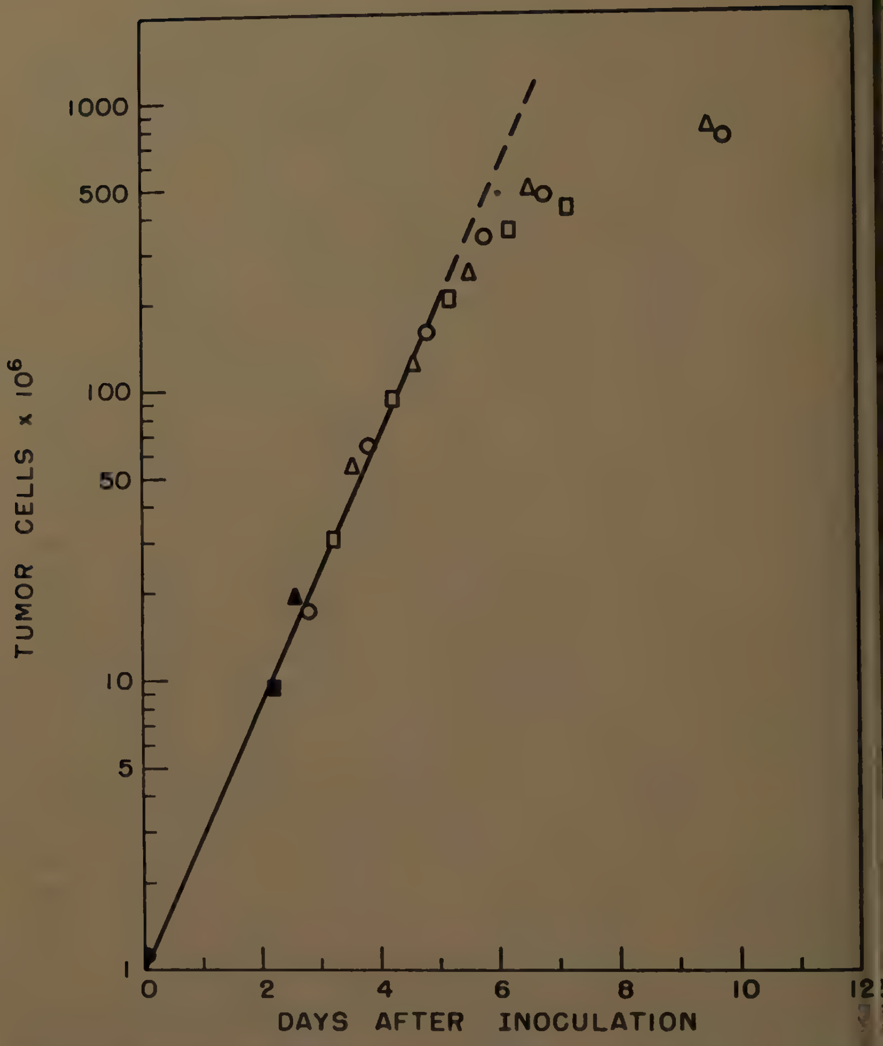

Figure 1. Exponential relationship between tumor cell numbers and time after inoculation. (The data for the three inoculum groups, designated by solid symbols, have been transposed to a common time axis. Re-printed from *Cancer Research*.[7])

by both expressions. Neither the exponential nor the cube root accounts for the entire growth pattern.

Doubling time during the log phase has been estimated as approximately 16 hours for the Krebs-2 ascites,[7] and 21 hours for the Ehrlich ascites[13] with comparable inocula. This difference in growth rate is also borne out by comparison of the slopes (cube root versus days) during the period of progressively increasing mean generation time.[7] The significance of the over-all comparison of growth rate is strengthened by the fact that the latter is based on Ehrlich ascites tumor data derived from another source.[6] It is noteworthy that the

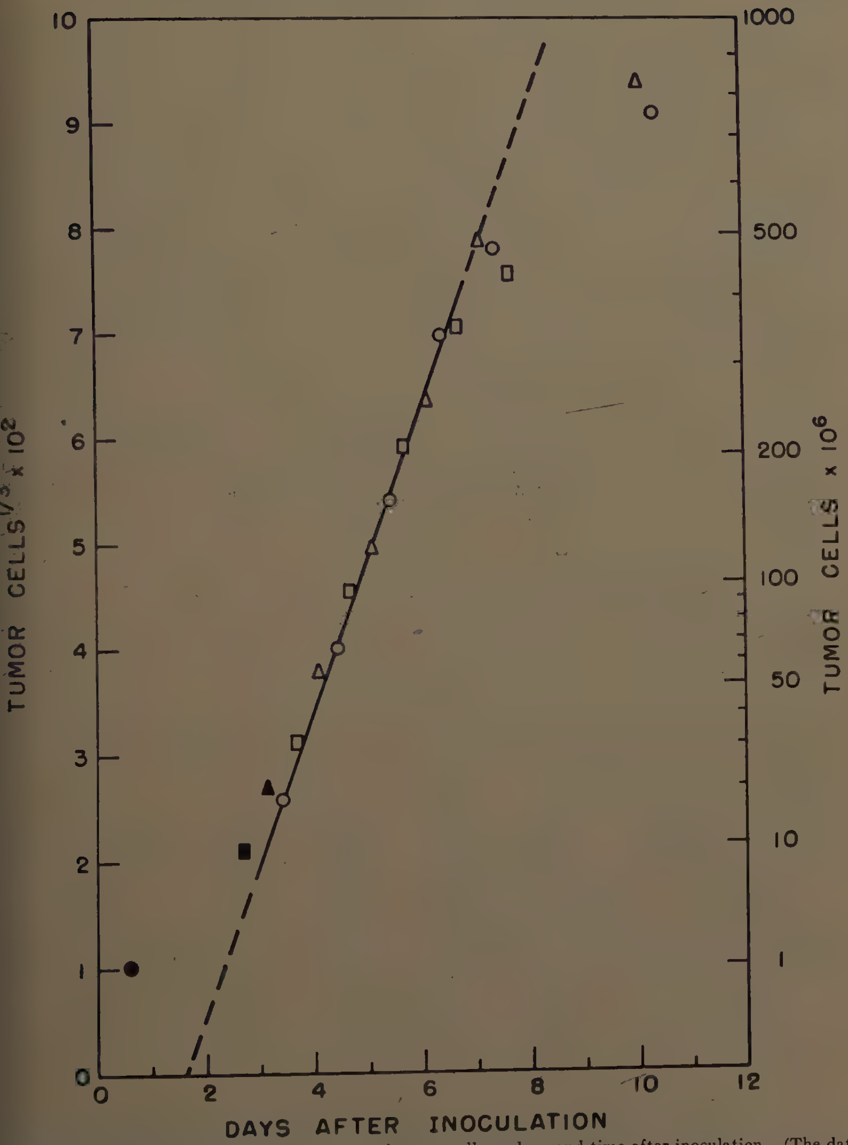

FIGURE 2. Relationship between cube roots of tumor cell numbers and time after inoculation. (The data for the three inoculum groups, designated by solid symbols, have been transposed to a common time axis. Reprinted from *Cancer Research*.[7])

doubling time for several ascitic lymphomas of mice varies between 5 and 10 hours for a given inoculum dose.[3] This brief mean generation time may be a reflection, in part at least, of their small cell volume, *e.g.*, *ca.* 400 cu.μ for the EL4 lymphoma versus 2000 cu.μ for the Ehrlich carcinoma. This conclusion is perhaps supported by the fact that the doubling time for a given inoculum of the EL4, DBA, and 6C3HED lymphomas varies directly with their mean cell volume (TABLE 1). Although a simple relationship of this sort does not obtain for the entire spectrum of ascites tumors, it may be recalled that the generation time for *Amoeba proteus* is also related to its initial mass.[16]

It is appropriate to consider briefly the pattern of ascites tumor growth in terms of the stem cell hypothesis advanced by Hauschka.[17] According to this concept, only a fraction of the total tumor cell population is capable of normal and regular mitosis. The constancy of a certain chromosomal modality or "germ-line" through many transplant generations of the Ehrlich and Krebs-2 ascites carcinomas and the DBA lymphoma is adduced as supportive evidence for this view.[18] Of interest is Richard's report[19] that the aneuploidic cells present in interphase and metaphase do not undergo or complete mitosis and, therefore, may constitute the nonstem cell portion of the population. The idea of stem cells is not incompatible with ascites tumor growth curves if it is assumed that the same proportion of stem and nonstem cells exists in the inoculum as in the growing tumor. If the large majority of tumor cells were incapable of division, however, it would be necessary to postulate an intermitotic time of only a few hours for such tumors as the Krebs and Ehrlich ascites.

Indirect Criteria of Tumor Growth

Criteria other than cell enumeration have been used from time to time to evaluate one or another aspect of ascites tumor growth. These include survival time, body weight change, percentage of takes as a function of inoculum size, percentage and concentration of free tumor cells in the ascites, and mitotic rate. As will be shown, such indices do not necessarily reflect the course of cell multiplication. They may provide useful information, however, under certain conditions.

Survival time, while somewhat dependent on the inoculum dose,[2] is at best only an approximate indicator of tumor growth. Studies with the Krebs-2 ascites tumor of mice reveal that differences in survival time may be considerably in excess of the time required for attainment of equivalent cell numbers. It has been shown, for example, that 50 per cent mortality may occur by the sixth day with an inoculum of 10^7 cells and, on the 17th day, with an inoculum of 10^6 cells, yet the corresponding cell growth curves are separated by only 2.3 days. Such differences may perhaps be related to the increased capacity of the host to adjust to the stress imposed by the slightly more prolonged development of a comparable ascitic volume.

A relationship between body-weight gain, ascitic volume, and tumor cell number has been inferred by Lettré[20] from analysis of the weight curves of tumor-bearing mice. Klein,[2] on the other hand, believes that the weight response is too variable for use as a quantitative index of tumor development. It has been shown more recently by Patt and Blackford[7] that body-weight change is a complex function of tumor growth, reflecting not only the ascites but also the associated anasarca and wasting. Prediction of the number of tumor cells from either the ascitic volume or the body-weight change is subject to a large error of estimate.

The early reports of a critical cell number for ascites formation are challenged y the more recent success of Hauschka,[17] Kaziwara,[21] and Yoshida[22] in growng clones and sublines from isolated single tumor cells. It is becoming inreasingly apparent that the percentage of takes with inoculum dose is a ariable parameter. Klein[23] has observed a striking decrease in critical cell umber with time for a given ascites tumor.

There is a tendency on the part of some investigators to use the percentage nd concentration of tumor cells in the ascites or the mitotic index as a measure f tumor growth.[24, 25] It is appropriate to point out that such indices may be nisleading. The percentage of tumor cells may be relatively constant during he major part of the growth curve. The concentration of tumor cells is also easonably constant in many instances. Ascitic volume, for example, has een shown to increase linearly with tumor cell number in the Ehrlich and Krebs-2 tumors.[5-7] A more complex relationship exists, however, in some scitic lymphomas. In these tumors, cell concentration may show a rapid ncrease followed by a continuous decrease, although the total number of free ells is increasing.[3] Evaluation of population growth in terms of the mitotic ndex is possible only if the duration of mitotic time is known and an assumpion is made concerning the linearity of the increase in number of cells. It nay be recalled that there is actually little consistent change in the mitotic ndex for the Krebs and Ehrlich ascites tumors until the terminal period. For he most part, differences that are observed are not statistically significant.

Summary

A quantitative approach to ascites tumor growth may be made by deternining the total number of free tumor cells in the peritoneal cavity. The nethods that have been used for this purpose are discussed and their limitaions are noted. Results obtained with several mouse ascites tumors reveal hat tumor development is relatively uncomplicated initially and may be haracterized in most instances by an exponential increase in the number of ells with little lag. The initial phase is followed by a period of progressively leclining growth toward an asymptote. The leveling phase reflects a complex ituation to which many physical and chemical factors may contribute. The ver-all pattern appears to be a function mainly of the total number of free umor cells and resembles the growth of populations generally.

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Discussion of the Paper

Doctor Oscar W. Richards (*American Optical Co., Southbridge, Mass.*): Having no practical experience with ascites tumors, my discussion will concern population growth problems. Actively growing unicellular organisms continue to grow at the same rate when inoculated into a favorable medium as long as the medium remains effectively constant. The logarithmic growth curves of Doctor Patt demonstrate this fact very well.

On the other hand, previous speakers have stated that the early growth of other tumor strains may show a lag period, and growth may not occur unless the seeding is above a minimum number of cells. These conditions indicate that, for those strains, the peritoneal exudate is not an adequate culture medium. The lag period may be due to the inoculum not coming from a constantly (logarithmic period) growing population, or to a conditioning of the medium. Older cells may need to readjust before constant proliferation is possible. When the medium is not too unfavorable, a larger inoculum may change the medium by absorbing or neutralizing materials, carrying over some essential materials along with the cells, or the rapid death of some of the cells may liberate the materials into the medium that lessen its toxicity, or contribute essential nutrients.

With yeast, protozoa, bacteria, and other single-celled organisms, the preparation of the seeding is important, and it would be interesting to know more of the techniques used in ascites work. Washing the seed cells with a suitable medium may change their subsequent growth, as was demonstrated in studies on "allelocatalysis."

The first divisions of a population, especially with inocula from cultures that have completed the log period of growth, are often a labile period when the newly formed cells can develop enzymes that will permit growth in an otherwise unfavorable environment. This complication makes it difficult to know whether such changes arise under an environmental stress or whether mutants with these advantages were present in the seeding and only the latter proliferate.

The slowing of growth in a changing medium may be due to the accumulation of toxic products, the diminution of nutrients, or both. Also, is the mortality selective (*e.g.*, killing of the young cells before they attain resistance) or nonselective?

following the infection with virus. A typical differential cell count obtained in one of the experiments with Bunyamwera virus was as follows:

Control smear, *i.e.*, one prepared from the pool of peritoneal aspirates of mice carrying noninfected Ehrlich ascites tumors:

Cancer Cells	Histiocytes	Polymorphs	Lymphs
76%	16%	4%	4%

Smear prepared from the pool of peritoneal aspirates of mice bearing Ehrlich ascites tumors infected with Bunyamwera virus:

Cancer Cells	Histiocytes	Polymorphs	Lymphs
3%	47%	4%	46%

In addition to such a dramatic drop in the percentage of cancer cells in smears from Ehrlich ascites tumors infected with certain oncolytic viruses, there were other morphologic findings indicating cellular degeneration and necrosis. Those consisted mostly of karyorrhexis, karyolysis, cytolysis, and presence of and phagocytosis of debris by histiocytes. The mitoses were reduced in number or absent whenever oncolytic viruses were used in sufficiently large concentration to cause rapid destruction of malignant cells. In case of a highly oncolytic virus, however, such as Mengo virus, it was found in individual animals that the oncolysis was sometimes accompanied by a terminal increase in the number of mitoses when higher dilutions of this agent were used. In such cases, the appearance of oncolysis was delayed, and it was also observed that smears contained occasional epithelioid cells, thus indicating an attempt at the formation of the granulomatous type of lesion[3] (see PLATES I and II).

It should be stressed that, in case of Mengo virus, the use of exfoliative cytology provided the only conclusive demonstration of its interference with the growth of Ehrlich ascites tumor. The bioassay method could not be used for the evaluation of the effect of this agent upon the growth of subcutaneous inocula because the animals were dying of virus infection before any tumors developed.

Thus exfoliative cytology was instrumental in establishing the ascites tumor-virus system, which has been found to be a valuable tool in biologic research.

The following may be said about the general usefulness of exfoliative cytology for the study of ascites tumors: Papanicolaou's method of ether-alcohol fixation, combined with his polychrome stain, provides excellent preservation of cells, emphasizes nuclear details, and allows for good nuclear-cytoplasmic differentiation and cytoplasmic transparency.[4] The smears treated by this method provide suitable material for a relatively easy identification of various cellular elements. Because of those advantages, the Papanicolaou method may be used as an excellent, although not the simplest, routine fixing and staining procedure for smears from inocula used for passages of tumors. It is suitable for screening smears of ascites tumors for effects of therapy. It is an instructive method for laboratory workers concerned with normal and neoplastic cellular morphology of ascites tumors, and it is preferred by those who have used it for diagnostic procedures.

While the use of ether-alcohol fixation and Papanicolaou's stain are generally accepted in diagnostic cytology laboratories, exfoliative cytology, in a broad sense, is not restricted to the use of the above procedure. Simpler stains may be preferred by some investigators for the routine passages or screening of smears of ascites tumors. The relatively complicated polychrome staining procedure is worthwhile undertaking, in cases in which finding and recognition of malignant cells require utmost skill. This is not the case with most ascites tumors, leaving, perhaps, lymphomas apart. As a rule, in a widely used Ehrlich ascites tumor, for example, distinction between malignant cells and nonneoplastic cellular elements is very clean-cut and can be made by relatively inexperienced morphologists with the use of simple stains or phase-contrast microscope.

In case of the lymphoma ascites, such as 6C3HED, DBA lymphoma, lymphomas Nos. 1 and 2, the distinction between malignant and inflammatory cells is not readily perceived, regardless of the method of staining employed. The criteria used by exfoliative cytologists for the evaluation of the neoplastic nature of various cells of the lymphoid type are still in the process of making. This became apparent in the course of a study carried for the past four years by the author in cooperation with Ralph Engle, and lately also with John Ultmann from the Department of Medicine of Cornell University Medical College.[5, 6] Our investigation is based upon comparison of normal and intrinsic neoplastic cells in consecutive contact smears of the same lymph nodes stained by Wright-Giemsa and Papanicolaou's stains, respectively. Although we were able to identify several types of normal and abnormal lymphoid cells with Papanicolaou's stain in certain types of lymphoma, in others the diagnosis was based upon the predominance of a single-cell type rather than upon the recognition of the neoplastic features of individual cells. The effort involved in this type of study is worthwhile making for the sake of learning more about the distinguishing features of exfoliated lymphocytic cells in human body fluids examined routinely with Papanicolaou's stain. It is probably more practical, however, for those investigators who study lymphoma ascites to evaluate cells in smears stained by hematologic stains.

The workers who had opportunity to utilize successfully the cytologic method for the quantitative evaluation of effects of oncolytic agents upon ascites tumors may well appreciate difficulties connected with attempts to base studies of exfoliated cells in human secretions on a par-excellence quantitative basis. Differential cell counts in gynecologic smears, as well as in pleural and peritoneal effusions, have been made by several investigators. These attempts were usually aiming at the evaluation of the effects of established or experimental therapy upon exfoliated malignant cells. One of the difficulties encountered in such attempts is the necessity for an arbitrary classification of cells of uncertain nature. Other difficulties are the lack of suitable control material, frequent presence of only a small number of malignant cells, and their irregular distribution. A study of cells in effusions of several inoperable cancer patients subjected to experimental inoculation with the West Nile virus at the Memorial Hospital in New York were reported in a separate communication.[7]

Thus the use of differential counts for the evaluation of the effects of treatment upon exfoliated human malignant cells is of a more limited value than in the case of the evaluation of oncolysis in ascites tumors.

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Discussion of the Paper

Doctor George Klein (*Wallenberg Laboratory, Institute for Cell Research and Genetics, Karolinska Institutet, Stockholm, Sweden*): Diagnosis of neoplastic cells in ascites tumors presents a problem somewhat different from similar questions in human pathology. In typical ascites tumors, the percentage of neoplastic cells is very high and represents a nearly pure culture. We do not wish to make a diagnosis of neoplastic disease from a few isolated tumor cells or clusters of cells appearing on the background of a largely inflammatory exudate. For our experimental purposes, we should like to assess the exact quantitative proportions of normal and neoplastic cells, and the question is whether we can do this with any degree of accuracy.

In our experience, different ascites tumors are very different in this respect. Some of them contain tumor cells of a large size with large and hyperchromatic nuclei and often huge and heavily stained nucleoli. From the studies of Hauschka and Levan, we know that these neoplasms are characterized by heteroploid modal chromosome numbers. After some practice, one can recognize such tumor cells rather easily, since they differ very considerably from nontumorous exudate cells. In fact, one may learn to differentiate one ascites tumor line from another and, in certain cases, two cytologically different lines may be mixed and each type can be differentially counted. We have performed some such studies, mixing the MC1M and TA3 ascites tumors in equal proportions and establishing the growth curve of each cell type in A × C3H F_1 hybrids (representing a combination of both parental types). A similar experiment was performed on Ehrlich and E.L.4 in C57 × C3H F_1 hybrids. In both cases, the mixed tumors clearly inhibited the growth of each other as compared to controls growing only one tumor line. The final total neoplastic mass was equal to, or slightly larger than, what would have been produced by one of the tumors if grown alone for the same length of time and from the same inoculum. I shall not deal with these studies in detail. They would serve only to illustrate the possibilities offered by at least some of the heteroploid ascites tumors with morphologically well characterizable tumor cells. One prerequisite of such studies is, of course, a thorough study of the cell types that occur in the normal and inflammatory peritoneal fluid of the host species. Wet fixation, as used in the Papanicolaou method, is of great help in emphasizing cytological and, particularly, nuclear details. In a series of counts where 1000 Ehrlich ascites tumor cells were counted on identical slides by three different people, an agreement within ±3 per cent could be obtained.

A very different problem is presented by the ascitic variants of most lymphatic tumors. The small size of the usually diploid neoplastic cells makes an exact differential count almost impossible or highly arbitrary. Fortunately, most ascitic lymphomas provide nearly pure cultures of one cell type soon after inoculation, and normal host exudate cells are rarely present to any considerable extent. It would appear more permissible to neglect host-cell admixture in such lymphomas than in other ascites tumors and to use the total cell count for calculations.

The question arises as to the factors that determine the actual proportions of tumorous and nontumorous cells in a given ascites tumor. One factor is the absolute number of the tumor cells themselves. This is dependent on the number inoculated, on their multiplication in the fluid, their death rate, and the rate by which tumor cells are desquamated from solid tumors of the peritoneal cavity, if present. The peritoneal fluid of the normal, untreated C3H mouse weighing about 20 g. usually contains between 4 and 7 million free cells, mostly of the lymphocytic and monocytic type. If the number of tumor cells is small compared to this value, their proportion will be low even in the absence of every inflammatory reaction. When the total number of free nontumorous cells was followed during the growth of the Ehrlich ascites tumor, it was found[1] that nontumorous cells accumulate in direct proportion to tumor-cell multiplication and that the newly appearing exudate cells are added to the original pool. The mechanism of this phenomenon is quite unknown, although it is probably linked to the accumulation of the fluid, since the latter can be described by the same type of equation. No sudden inflammatory reaction can be found during any part of the growth cycle. This holds true only for the untreated tumor, however, and the situation becomes quite different if the tumor cells are damaged in some way. At least in the case of X rays, it might be suggested that the severe inflammatory reaction that is sometimes seen is not provoked by the radiation itself but by some products of the damaged tumor cells. The same X-ray dose may or may not provoke an inflammatory reaction in different ascites tumors, depending on the radio-sensitivity of the tumor cells. There seems to be a parallelism between the extent of tumor cell death and the intensity of the reaction.[2]

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PLATES

PLATE 1*

Note. All smears illustrated in PLATES 1 and 2 were fixed in ether-alcohol and stained by Papanicolaou's method. All photomicrographs were obtained with the same magnification (×600). Figures have been slightly reduced for publication.

FIGURE 1. Smear from noninfected control Ehrlich ascites tumor nine days after implantation. Malignant cells predominate.

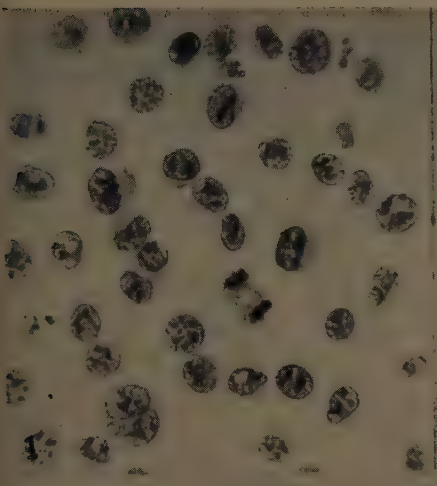
FIGURE 2. Smear from Ehrlich ascites tumor infected with Eastern Equine Encephalomyelitis virus. Malignant cells predominate.

FIGURE 3. Smear from Ehrlich ascites tumor infected with St. Louis virus. Numerous histiocytes and well-preserved malignant cells may be seen.

FIGURE 4. Smear from Ehrlich ascites tumor infected with Ilheus virus. Degenerated cells in karyorrhexis and well-preserved malignant cells are present.

FIGURE 5. Smear from Ehrlich ascites tumor infected with Bunyamwera virus. A single cancer cell and numerous inflammatory cells (mostly histiocytes) are shown.

* Reproduced from *Cancer Research*.[2]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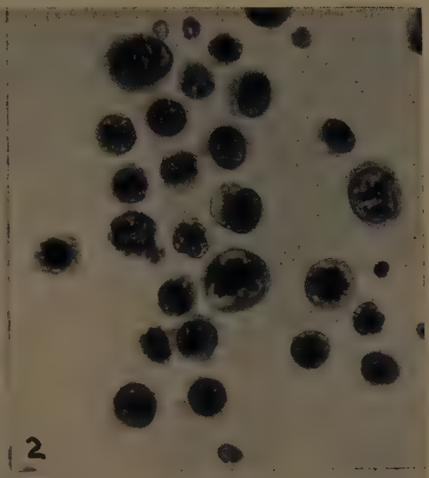
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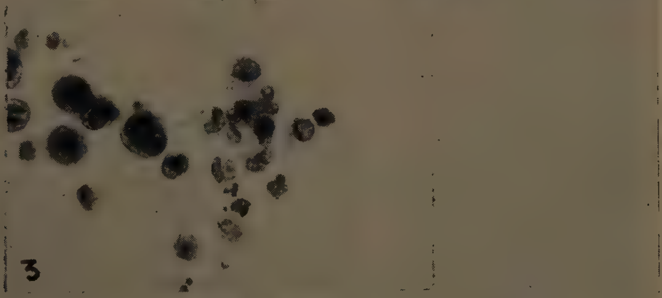
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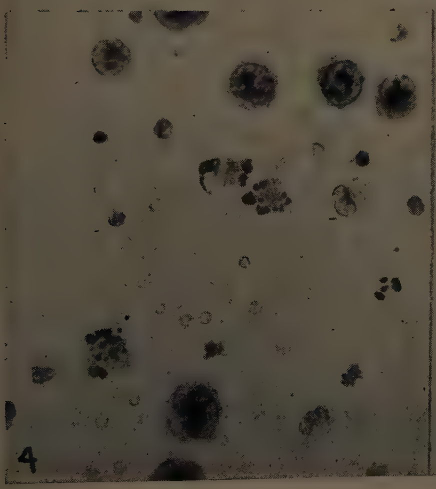
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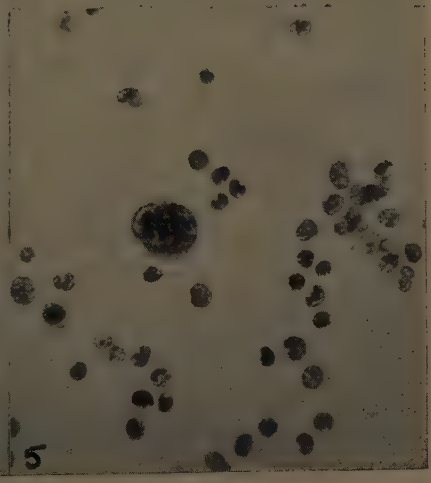
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PLATE II**

FIGURE 1. Ehrlich ascites tumor cells in a control smear.

FIGURE 2. Histiocytes containing phagocytic inclusions on the third day following inoculation of 10^{-8} dilution of Mengo virus.

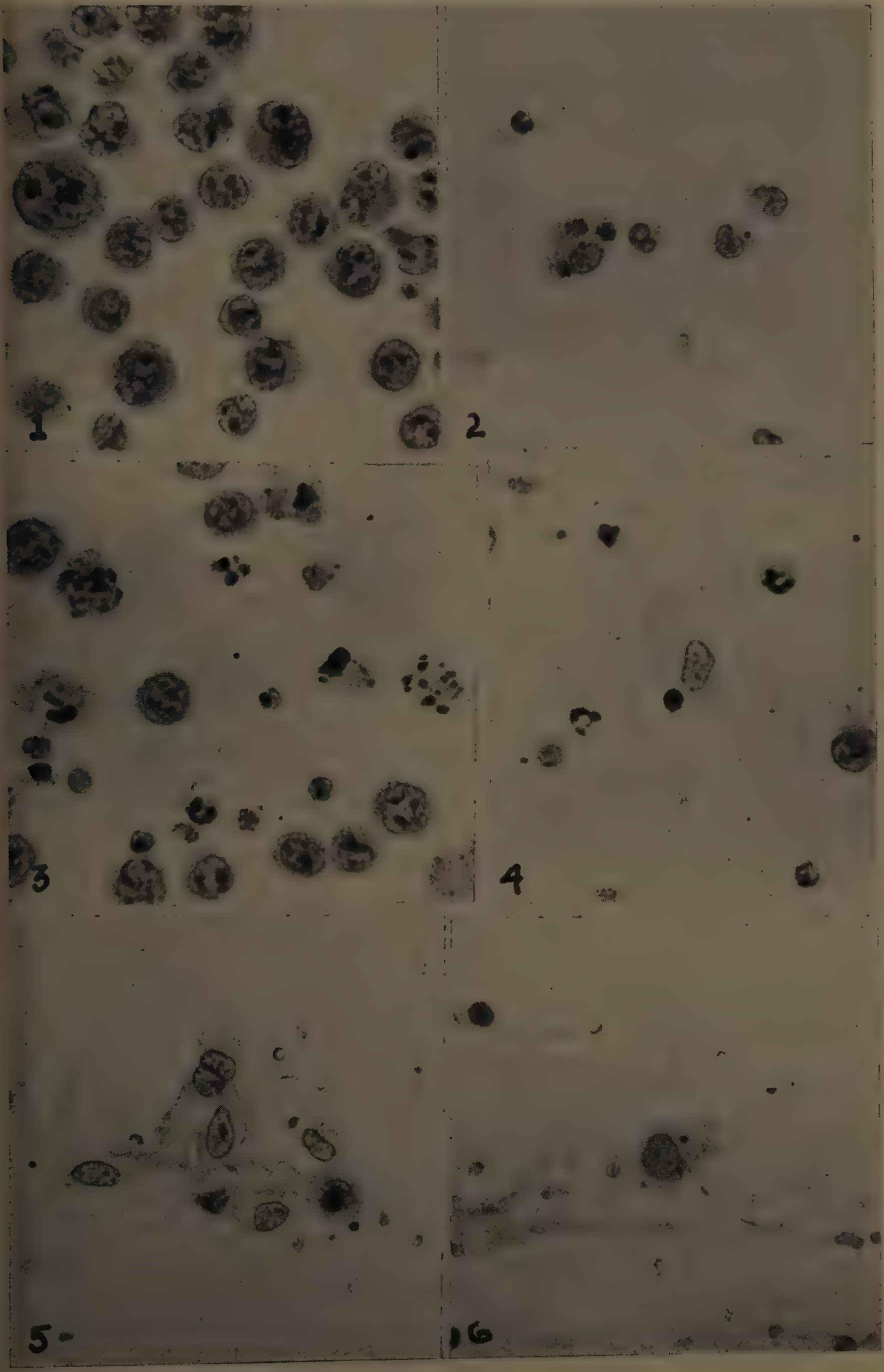
FIGURE 3. Numerous cells in karyorrhexis on the first day after inoculation of 10^{-2} dilution of Mengo virus.

FIGURE 4. A large epithelioid histiocyte found on the second day after inoculation of 10^{-2} dilution of Mengo virus.

FIGURE 5. A group of large epithelioid histiocytes seen on the third day after inoculation of 10^{-7} dilution of Mengo virus.

FIGURE 6. Unusual histiocyte similar to those illustrated in FIGURES 4 and 5 and containing cytoplasmic inclusions that favor the theory of its phagocytic nature.

** Reproduced from *J. Natl. Cancer Inst.*[3]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ELECTRON MICROSCOPE STUDIES OF ASCITES TUMOR CELLS*

By Cecily Cannan Selby,† John J. Biesele, and Clifford E. Grey
Sloan-Kettering Institute for Cancer Research and the Sloan-Kettering Division of Cornell Medical College, New York, N. Y.

Ascites tumor cells were chosen in our laboratory for early electron microscopic studies because they can be prepared relatively free of some of the gross preparative defects encountered in pieces of solid tissue. Variations in degree of fixation, dehydration, and embedding in different areas of even very small pieces of solid tissue, as a result of the slow penetration of perosmic acid (the best fixative for both light and electron microscopy) and incomplete and uneven polymerization of plastic embedding (as introduced by Newman, Borysko, and Swerdlow in 1949, the best embedding for ultrathin sectioning) cause cytological distortions that, although not obvious under the light microscope, are objectionable under the high magnification and resolution of the electron microscope. These may be avoided in working with free cell suspensions such as the ascites tumor cells, where all preparative solutions can be introduced directly into the suspending fluid (originally the ascitic fluid) bathing each cell. Thus the effects of preparative techniques are uniformly distributed among all the cells, and comparisons between various fixatives or embedding materials can be made with some confidence.

As preparative techniques have improved each year, increasingly more information has been gained concerning the submicroscopic cytology of ascites tumor cells. This information has also been useful in providing a base line in the study of other cell types. In what follows, the general method used in preparing ascites tumor cells for examination by electron microscopy will be outlined, the normal morphology of the ascites tumor cells will be described as seen in high-magnification study of thin sections, and some changes in morphology during mitosis and during various types of cellular degeneration will be discussed.

The Preparation of Cells for Microscopy

The ascitic fluid is removed from the living animal with a syringe and immediately mixed with the desired quantity of fixative. With ascitic fluids containing approximately 10 per cent cells by volume, we add an equal volume of a 2 per cent solution of osmium textroxide, or up to 10 volumes of a 1 per cent solution of fixative directly to the fluid. The osmium tetroxide solution was originally made up to 1 per cent in Locke-Ringer solution with a resultant pH of 7.1, but recently the Palade (1952a) acetate-veronal buffer at pH 7.3 has been used so that the techniques used with the solid tumors and ascites tumors would be identical. At the end of the desired period of fixation, most of the supernatant fluid is removed after slow centrifugation for about five minutes and replaced with wash water or isotonic salt solution. After another slow centrifugation, the supernatant fluid is again removed and the cells resuspended in another wash solution. Cells are commonly washed with three changes over one hour. The pellet following the final wash is resuspended in 70 per cent alcohol, and the cells are dehydrated by repeated centrifugation and resuspension in alcohols of increasing concentration with about 30 minutes allowed in each alcohol. Cells spun down from the second change of absolute alcohol are suspended in uncatalyzed methacrylate, centrifuged again, and transferred with the aid of a platinum spatula directly into capsules, in which they are immediately immersed in catalyzed methacrylate. The cells fall to the bottom of the capsule and are thus embedded at the tip of the capsule ready for sectioning. Polymerization is accomplished overnight in an oven at 45° C.

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The embedded cells are sectioned at 0.02 to 0.08 micron for electron microscopy in either of two microtomes designed and built in our laboratory (Grey and Biesele, 1953; Selby, 1955). One of these (Selby, 1955a) is readily adjusted to section the blocks also at one micron for parallel light microscopic studies.

Normal Morphology of Ehrlich Ascites Tumor Cells

Mice bearing Ehrlich ascites tumors were kindly provided by Doctor K. Sugiura and by Doctor Charlotte Friend, both of this Institute, from later transplant generations of an Ehrlich line, presumably hypotetraploid, originally furnished Doctor Sugiura by Doctor George Klein (Sugiura, 1953).

Phase-contrast photomicrographs of one-micron sections of Ehrlich ascites tumor cells have been published by Selby *et al.* (1954). Considerable cytological detail is evident in these osmium-fixed cells, which would be obscured in thicker sections or in cells treated with less faithful fixatives, *e.g.*, mitochondria and coiled nucleolar structure.

Electron micrographs of sections cut at approximately 0.05 micron of methacrylate-embedded pellets of Ehrlich ascites cells show an absence of close-packing of cells in the pellets and a good preservation of circular cell profile and of cell organization. The many slow centrifugations following fixation apparently do not affect the internal cell organization, because the nuclei are centrally located, the central zone is dense, and the ectoplasm is clear. Specimens taken about one week after an inoculation with one million cells are characterized by cells with few fat droplets or vacuoles, one or more nucleoli with prominent filamentous structure, little dense chromatin, and by a relatively high percentage of mitotic figures.

The general organization of these cells is illustrated in FIGURE 1. Many vesicles and granules of various sizes occur in the cell center, mitochondria and double membranes are concentrated between this region and the ectoplasm and are frequently radially oriented, while the ectoplasm is usually free of large cytoplasmic components. Lipid droplets appear as extremely dense and osmiophilic crenated spheres, whose density and degree of crenation increase with the fixation. This cell was from a specimen centrifuged and washed with saline during a five-minute period between removal from the host and exposure

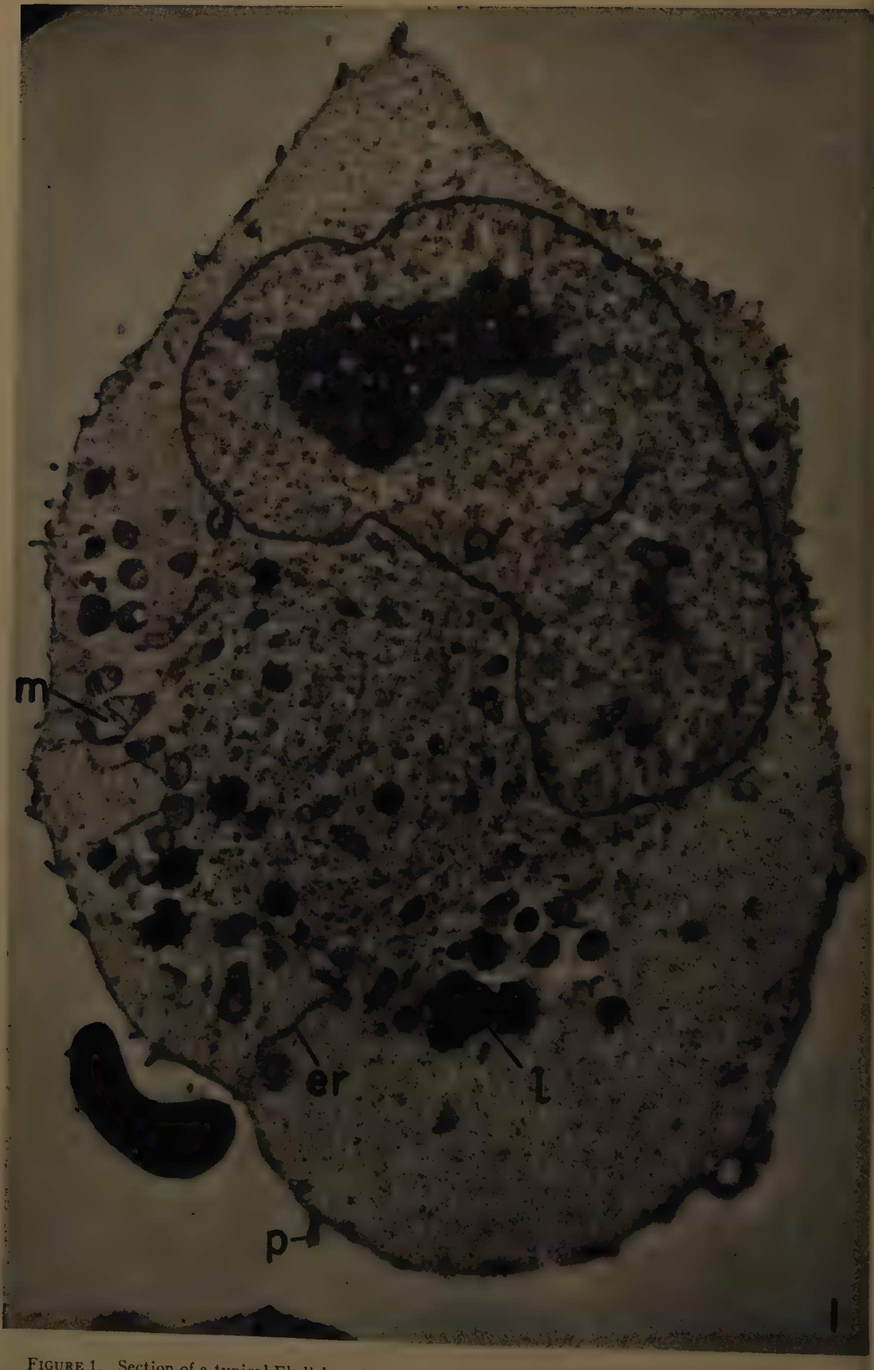


FIGURE 1. Section of a typical Ehrlich ascites tumor cell showing eccentric nucleus with one nucleolus, cell center with dense accumulations of vesicles and granules, mitochondria (m), lipid droplets (l), and endoplasmic reticulum (er) in an area surrounding the cell center, ectoplasm empty of large particles and the cell membrane with many pseudopods (p). ×3,870.

o fixative. The effect of this delay in fixation may be seen in the swelling of he mitochondria and slight distortion of the cell membrane resulting in shorter cytoplasmic projections or pseudopods than are found in optimally preserved cells.

Constant-diameter projections of the cell membrane that may be called pseudopods are found along the surface of every ascites tumor cell. They are believed to be a property of the living cell, because they have been observed by many authors in sections of various cell types bordering a cavity or fluid (Selby, 1955) and, in this study, along the surface of ascites tumor cells prepared as smears (rather than embedded for sectioning) after no or differing fixation. The quantity and size of pseudopods in smear preparations of these cells were found to be minimal in cells suspended in hypotonic solutions. Distinction should be drawn between these fine projections with a predominantly constant diameter of 0.1 micron and the gross bubbling or membrane defects visible in deteriorating cells under the light microscope. These fine pseudopods would not be visible by conventional light microscopic techniques.

The cell membrane and some cytoplasmic components are shown at higher magnifications in FIGURE 2. Here it is seen that the submicroscopic particuates present throughout the cytoplasm are not associated with the cell membrane or mitochondrial membranes, and thus occur freely except when they are lined up along the outer wall of certain tubules, parallel membranes, or vesicles, as described in other cell types by Palade (1953b, 1955a) and by other workers (Porter, 1954a; Sjöstrand and Hanzon, 1954b; and Palay and Palade, 1955). These tubular profiles (*cf.* Dalton, 1951) appear identical to those described in other cells as the endoplasmic reticulum (Palade and Porter, 1952 and 1954) or ergastoplasmic structures (Bernhard *et al.*, 1952; Weiss, 1953; Bernhard *et al.*, 1954; and Howatson and Ham, 1955b). In view of the cell types in which these components are most prevalent, it has been suggested that their presence is somehow associated with secretory and synthetic activities (Palade and Porter, 1952; Porter, 1953; and Weiss, 1953). Palade (1955b) has recently proposed that, in macrophages at least, the channels of the endoplasmic reticulum may arise from the pinching off of areas of the cell membrane between pseudopods. In the Ehrlich carcinoma ascites cells, however, canals or vesicles of the reticulum have not often been observed close to the cell membrane, nor does the cell membrane commonly show any invaginations between the pseudopods. Thus, it is probable that these cells do not have the same or as active a mechanism as macrophages for exchange of material with the surrounding medium (phagocytosis and pinocytosis). Although the ultrastructure of normal mammary cells has not yet been reported, such cells would be expected to have a highly developed endoplasmic reticulum if the latter is related to secretory and synthetic activity. The presence of this component in the tumor cells may therefore reflect their origin as mammary cells (Loewenthal and Jahn, 1932). If this is so, the development of the endoplasmic reticulum in the tumor cells is less than would be expected of active mammary cells and may therefore reflect a loss of function. The finding here is paralleled by those of Howatson and Ham (1955b) and is analogous to that of Porter

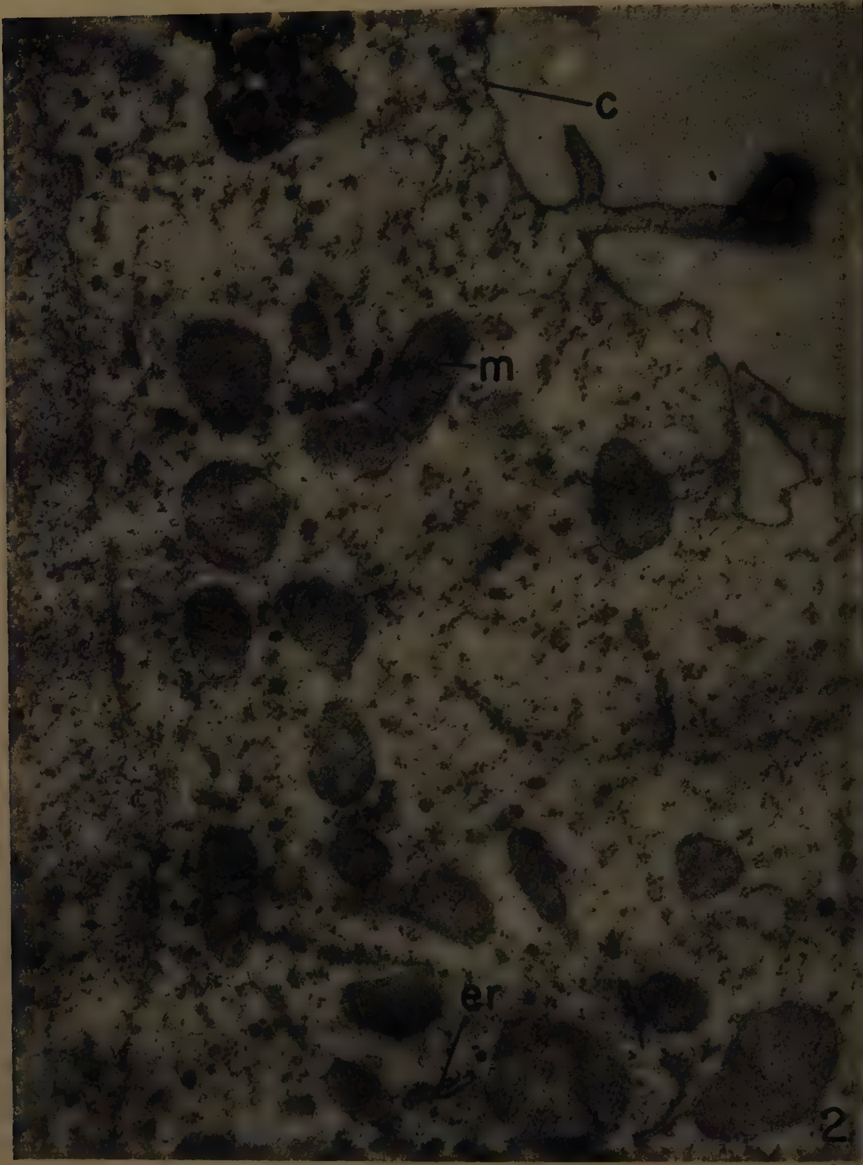


FIGURE 2. High-magnification view of an ultrathin section through a portion of an ascites tumor cell. T nuclear membrane runs along the left-hand side of the micrograph. Mitochondria (m), cell membrane (c), a endoplasmic reticulum (er) with attached submicroscopic particles are indicated. ×35,100.

(1954a), who noted that neoplastic cells have endoplasmic reticulum less regula in form and disposition than do their neoplastic counterparts.

Mitochondria, as shown here, have the usual internal structure with interna membranes or cristae (Palade, 1952b) that may be discontinuous but, contrar

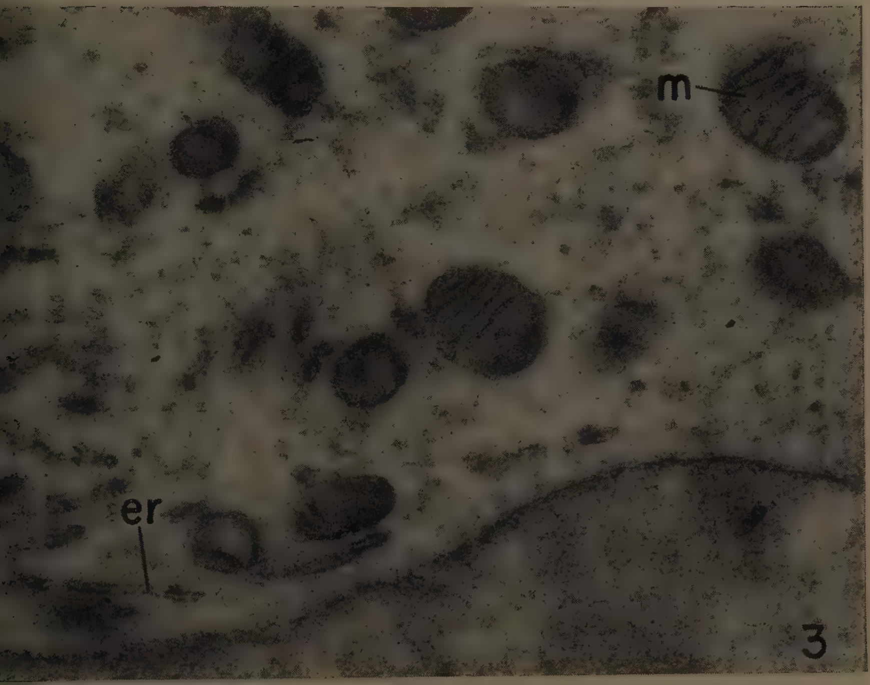


FIGURE 3. View of a thicker section through cytoplasm to show intact mitochondria (m) with internal membranes and dense matrix and portions of endoplasmic reticulum (er). Nuclear membrane runs along the bottom half of the micrograph. ×35,100.

to Palade, are usually continuous across the mitochondrial cavity as in FIGURE 3. Distortions in these internal membranes are often observed but may be due to imperfect preservation. There are fewer internal membranes per mitochondrion in these tumor cells than in the kidney, for example (Palade, 1953a; Sjöstrand and Rhodin, 1953; Rhodin 1954), and there is an irregularity to their arrangement inside the cavity. The over-all shape of the mitochondria is also very variable, with branched forms frequently found, as would be expected of cells with much cytoplasmic movement. Although no accurate measurements have been made, our observations are in accordance with Sjöstrand's conclusion (1954) of mitochondrial differentiation according to cell type.

A portion of the cell center is shown at higher magnification in FIGURE 4. It contains series of parallel membranes and granules or vesicles, which may be distinguished from the endoplasmic reticulum by the absence of attached submicroscopic particulates. The many vesicles are of variable size. By virtue of their predominantly circular, rather than oval, cross-section, and because of their quantity as compared to the number of longitudinal parallel double membranes, they may be interpreted as actual vesicles rather than as cross-sections of tubules. This part of the cell center resembles the structures described as the Golgi apparatus in the principal cells of the duodenum (Dalton, 1954), the epithelial cells of the epididymis (Dalton and Felix, 1954), and the

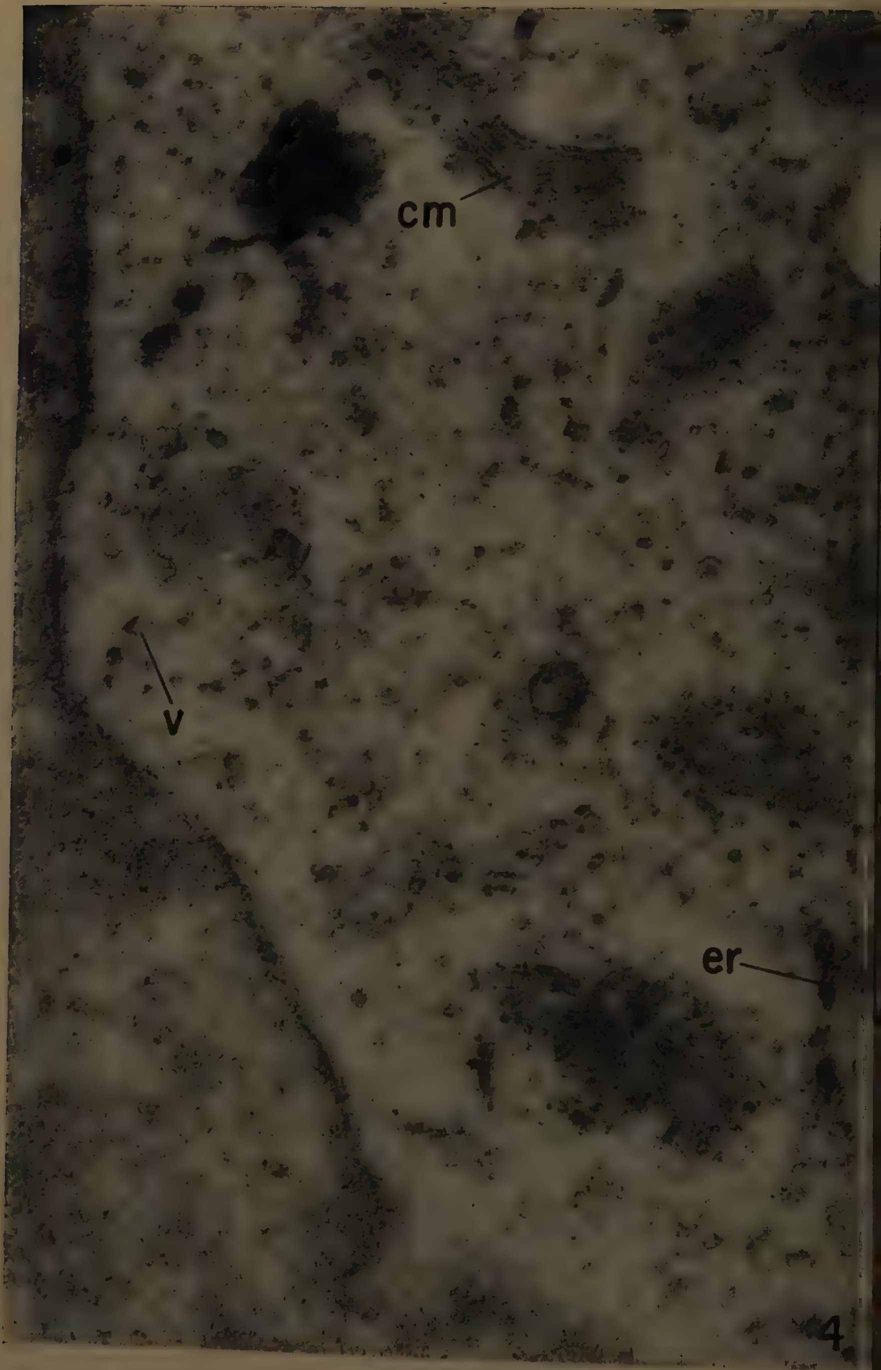


FIGURE 4. Section through the cell center of an ascites tumor cell. The nuclear membrane runs along th left-hand side of this view. The endoplasmic reticulum (er) has submicroscopic particulates attached to it wall whereas the membranes (cm) and vesicles (v) of the cell center do not. ×35,100.

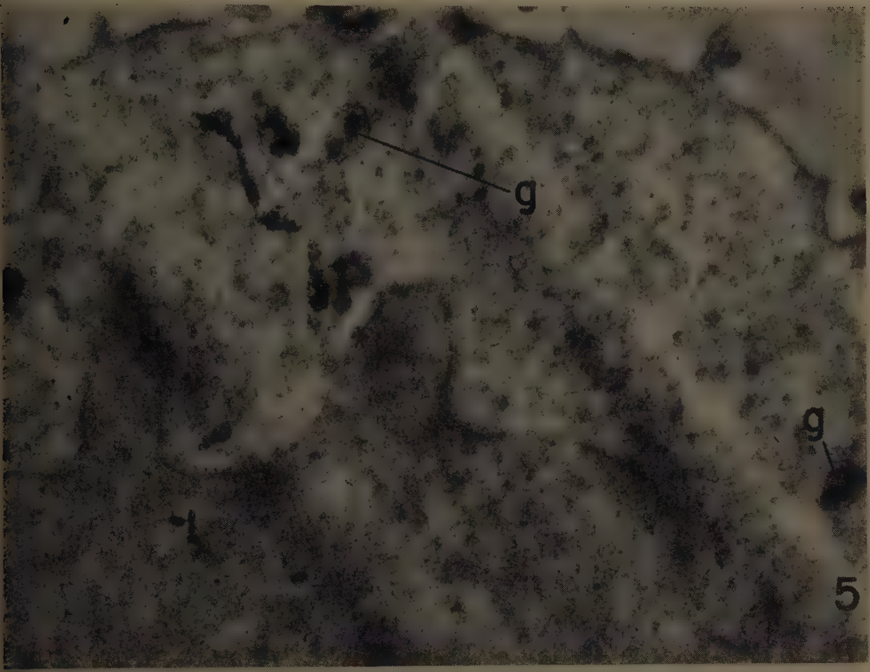


FIGURE 5. Section through a portion of an ascites tumor cell to show the unidentified granules (g) with surrounding vesicle found in these cells. ×15,210.

exocrine cells of the pancreas (Sjöstrand and Hanzon, 1954a, 1954c). In agreement with the statement made by Doctor Dalton in his discussion of this paper, however, we must also state that our ascites tumor cells have lacked the very large vacuoles associated with the Golgi apparatus of active secretory cells, as described by Dalton and Felix (1954) and by Sjöstrand and Hanzon (1954c). Rather, the structures in question in the ascites tumor cells resemble more closely the agranular reticulum described in various nerve cells (Palay and Palade (1955) and the tight piles of agranular membranes seen by Palade and Porter (1954) in the centrosphere region of macrophages.

Some of the granules found in the cell center may be noted occasionally elsewhere, although their incidence is so low that cells, such as that of FIGURE 5, where several are evident, are rare. The granules are distinguished by their size and enclosing vesicle or membrane. The dense central part is about 0.1 micron in diameter, and thus these granules may correspond in size and density to the granules described by Porter and Thompson (1947) and by Selby and Berger (1952) in the periphery of cultured tumor cells. Harel and Oberling (1954) have described their occurrence as the ultrachondriome in normal cells found in body fluids. There are not yet enough data concerning cytoplasmic components of this type in sectioned or cultured cells to discuss further their identity. Porter and Kallman (1953) describe dense granules of the same size in sectioned rat sarcoma cells. One of us (C.C.S.) has observed granules with

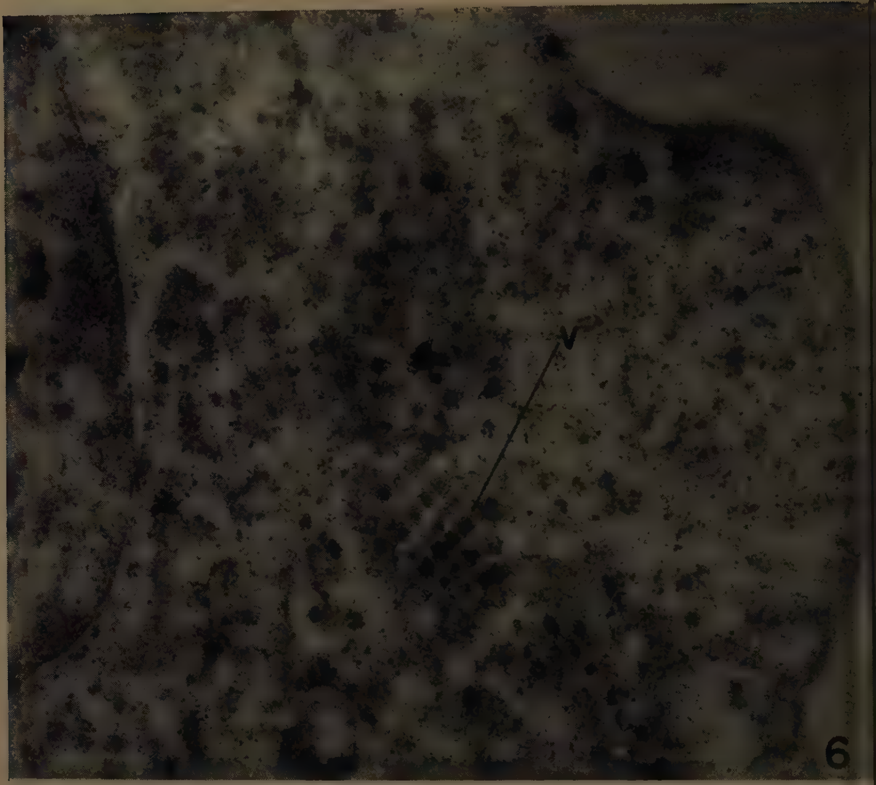


FIGURE 6. Ultrathin section of a cell containing the unidentified viruslike particles (v) occasionally foun in tumor specimens. The cell membrane runs along the right-hand side and the nuclear membrane along th left-hand side of this view. Submicroscopic particulates are distributed throughout the cytoplasm and are line up along the outer nuclear membrane. ×32,500.

a surrounding vesicle, similar to those described here in sarcoma 180 soli tumor cells, and has failed to see them in normal mammalian epidermis.

Other unidentified granules also found in the Ehrlich ascites tumor cell have been described by Selby *et al.* (1954). They are illustrated in FIGURE 6 which also shows small, dense, submicroscopic particles and a portion of th nuclear membrane perpendicularly sectioned. The larger granules in questio are readily recognized by the fact that they occur in very regular, often hex-agonal array and have a constant outer diameter of 580 Å. with a dense inne core about 300 Å. in diameter. When they do appear in a given ascites tumo specimen, their incidence is so high that they may be seen in about 20 per cen of the cell sections, or perhaps in a majority of the cells. They have bee completely absent from about 90 per cent of the specimens examined. Simila masses of particles, in equally regular array, have been seen in mitotic tumo cells and in leucocytes, as well as free in the ascitic fluid. Their incidence i otherwise healthy resting and mitotic cells, as well as in degenerate cells, an their all-or-none pattern of incidence among ascites specimens have led us t

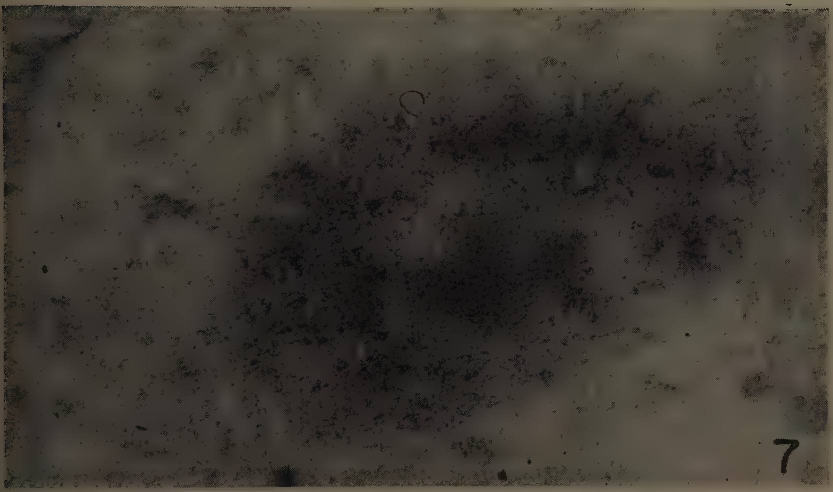


FIGURE 7. Tangential section through a nuclear membrane showing the pores revealed when the angle of section is optimal. ×35,100.

the conclusion that the particles represent some form of viral contaminant present in the mice from which the ascites was taken. Nothing further is known about the properties of these particles, because an unfixed specimen of Ehrlich tumor ascites known to contain them has never been studied, and they are recognized solely by their osmiophilia and could conceivably pass unnoticed in the usual histological preparation. These particles have not yet been seen in our several studies of mouse sarcoma 180 or normal mouse tissues. It may be noted that Gieseking and Schümmelfeder (1955) failed to find viruslike particles in the particular Ehrlich carcinoma cells that they studied.

An image similar to these particle arrays but easily distinguished by dimensions and other characteristics is that of the nuclear membrane in tangential section. Such a section is shown in FIGURE 7. The pore diameter and the interpore space have the same diameter as described by other authors in the oöcyte (*e.g.*, Gall, 1954). Rhodin (1954) was perhaps the first to point out nuclear membrane pores in mammalian material. In kidney cells of the mouse, the pores have a diameter similar to those illustrated here. Pores in the nuclear membrane have also been illustrated in sections of the Rous sarcoma by Gaylord (1955). The viruslike particles Gaylord found associated with sarcoma cells in two of three Rous tumors closely resemble those found in our Ehrlich carcinoma cells.

Within the nucleus, still more particulate material may be discerned. In FIGURE 8, which is of a section of specimen that had been fixed for 30 minutes, and therefore perhaps so long that considerable amorphous matrix was removed (*cf.* Porter and Kallman, 1953), the nucleus contains particulates of the same size and density as those in the cytoplasm. This resemblance has also been pointed out by DeRobertis (1954). These particles in the nucleus do not seem to follow any particular pattern or to be associated with any other

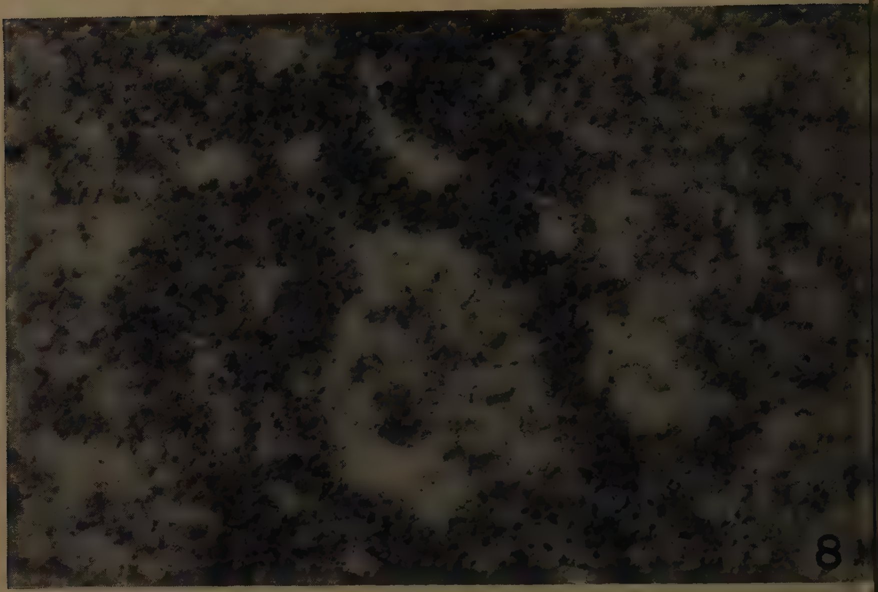


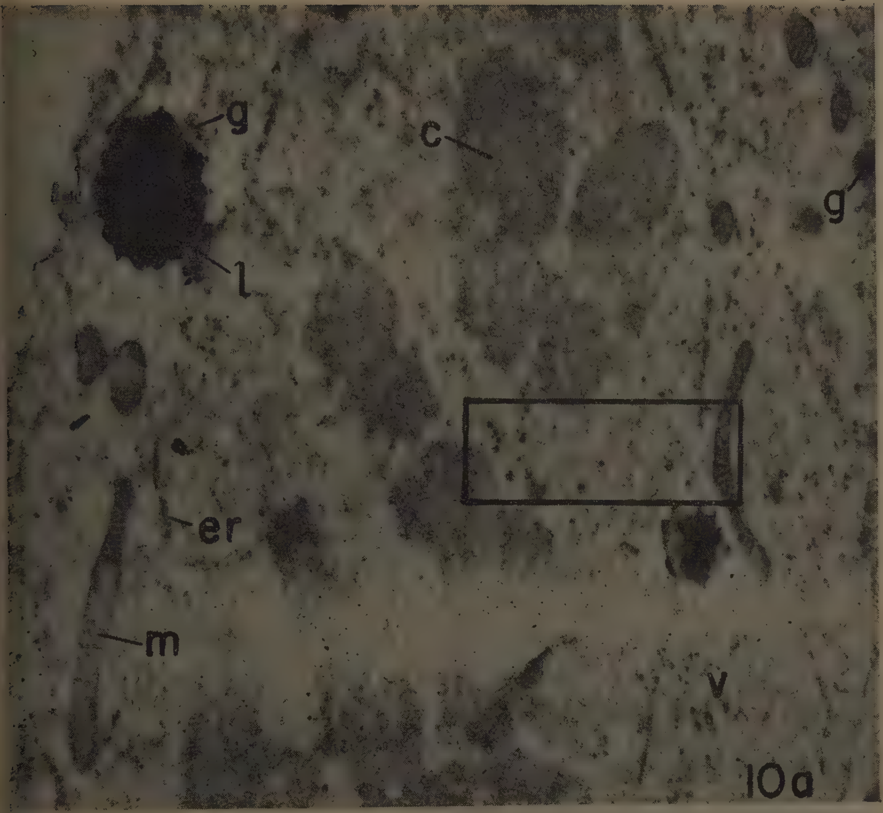
FIGURE 8. High magnification view of a portion of an ultrathin section through the nucleus. The dense accumulation of submicroscopic particulates in bands running through the center of this micrograph constitute part of the nucleolus. ×42,120.

structure except some very fine filaments that often appear to join them to each other. It remains for future work to determine to what extent this structure reflects a particulate and fibrous constitution of the living nucleus and to wha extent it reflects simply the action of osmium tetroxide on a protein and nucleo- protein gel. Bahr (1954) has found that, although nucleic acids are inert to- ward osmium tetroxide, histone from ox thymus is agglutinated as it reacts vigorously to reduce the osmium tetroxide to lower oxides.

In the nucleolus, these particulates may simply be arranged in a somewhat better organized close-packing, as Porter (1954a) has suggested. The nucleolar structure is especially prominent in nuclei showing so little granular chromati that at low power the nucleus appears to be homogenous with one or more nucleoli included. In such nuclei, the coiled nulceolar structure (Borysko and Bang, 1951) may be seen even in phase micrographs of thick sections, as shown in FIGURE 3 of Selby *et al.* (1954). This type of nucleus occurs in young ascites specimens during the vigorous growth phase following implantation and before much cytoplasmic fat and vacuolization appear. Nuclei with considerable granular chromatin have less density in the background matrix.

Degenerative Changes

Beside nuclear changes, other characteristics of "old" cells are shown in FIGURE 9. There are many cytoplasmic lipid droplets, and cytoplasmic vacuoles are often so large that the appearance of the familiar "signet ring" cell results (Seeger, 1937). It is of interest that there may be pseudopods



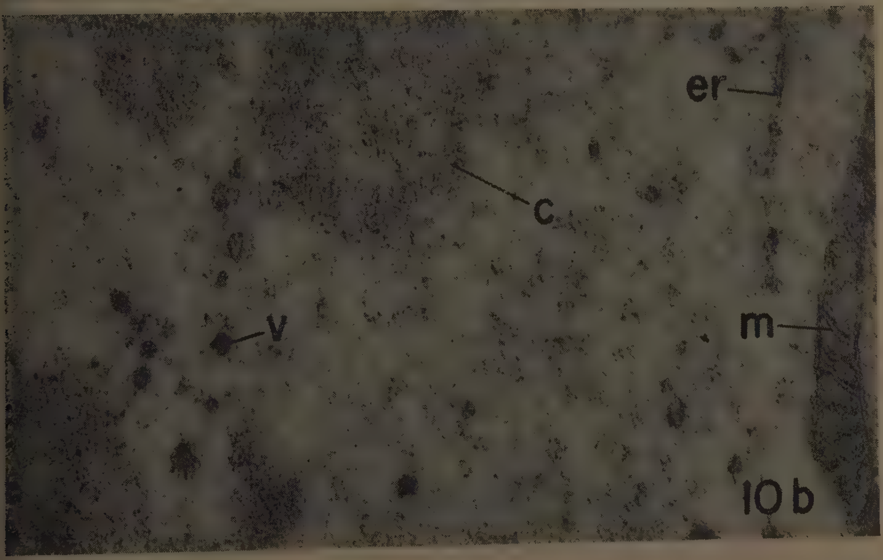


FIGURE 10. a. Section through an ascites tumor cell in early metaphase. Lipid droplet (l), mitochondria (m), cytoplasmic granule (g), endoplasmic reticulum (er), vesicles (v) of the cell center and chromosomes (c) are indicated. A knife mark runs along the lower half of this view, and sections portions of a chromosome and a mitochondrian particularly thinly. ×13,500.

FIGURE 10. b. Enlargement of indicated portion of FIGURE 10a showing mitochondrial internal structure (m) endoplasmic reticulum (er) with attached submicroscopic particulates, chromosome material (c) and vesicles (v) of the cell center. ×42,120.

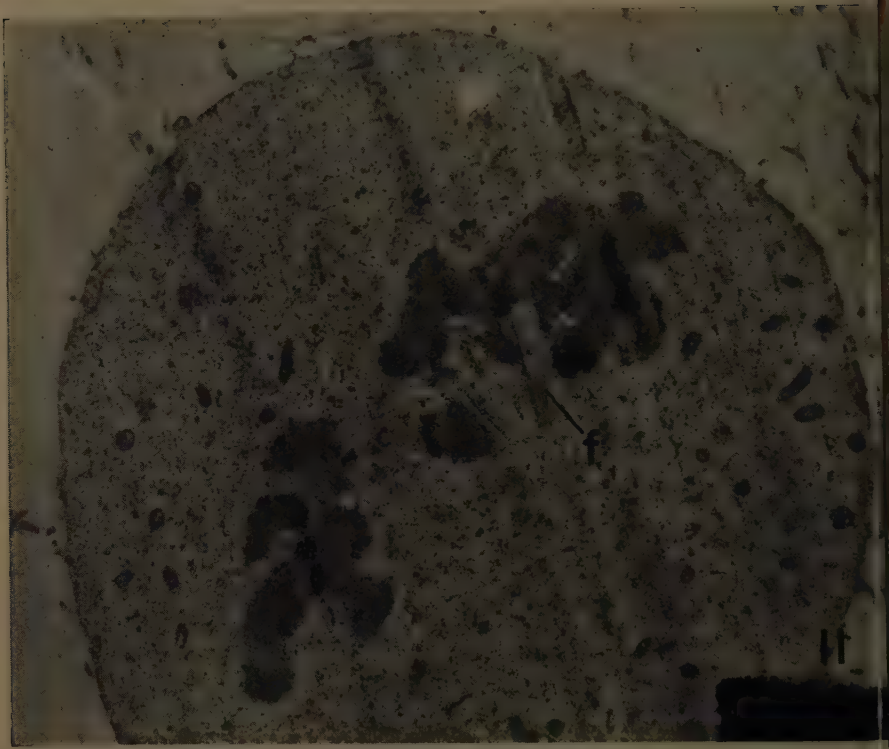


FIGURE 11. Low magnification of a portion of a cell in anaphase. Filaments (f) grouped together resembling chromosomal fibers are indicated as they appear in this particular angle of section. ×6,562.

branes with occasional long interruptions. The two membranes are more closely apposed than is the usual case with the membrane profiles of endoplasmic reticulum, and they may well be the remnants of the double nuclear membrane. In thin sections of a rat tumor, Porter (1954b) has noted that the endoplasmic reticulum is less prominent in prophase and metaphase, and that the small vesicles and granules of the cell center become widely distributed.

In anaphase, fine submicroscopic filaments become visible as they are grouped into bundles resembling the light-microscopic picture of chromosomal fibers (FIGURE 11). These have often been visualized in sections of cells at anaphase (*cf.* Selby, 1953a) but have not been observed in the spindle area in other stages of mitosis, except rarely in telophase cells. Porter (1955) has followed spindle and chromosomal fibers, appearing as condensations of fibrous material around a pair of filaments or tubules, through anaphase and early telophase. The region between the separating chromosome groups in dividing Ehrlich cells contains vesicles, presumably originating from the cell center, which are aligned predominantly into rows parallel to the spindle axis. Mitochondria and endoplasmic reticulum remain marginated throughout anaphase and early telophase. The mitochondria and endoplasmic reticulum return to the positions and orientations more characteristic of the resting cells by the time of the final constriction into two cells, as the nuclei are reconstructed and the Flemming midbody becomes visible (*cf.* FIGURE 24, p. 87, from our material in DeRobertis, Nowinski, and Saez, 2nd edition, 1954). By this time, vesicles are again in the cell center, the ectoplasm is clear, and filaments of the spindle are restricted to the division collar itself.

It is noteworthy that cell sections such as are illustrated here reveal no internal structure in the chromosomes. It may well be that the chromosomes are incompletely fixed by the osmium tetroxide to which the cells are subjected. Bahr (1954) remarked on the lack of reaction between nucleic acid and osmium tetroxide. Chromosome fixation is not improved by following osmium tetroxide with formalin, although this procedure does prevent a certain amount of washing out of cellular matrix following osmium fixation, as all cellular components have the same form, but the background of nucleus and cytoplasm is

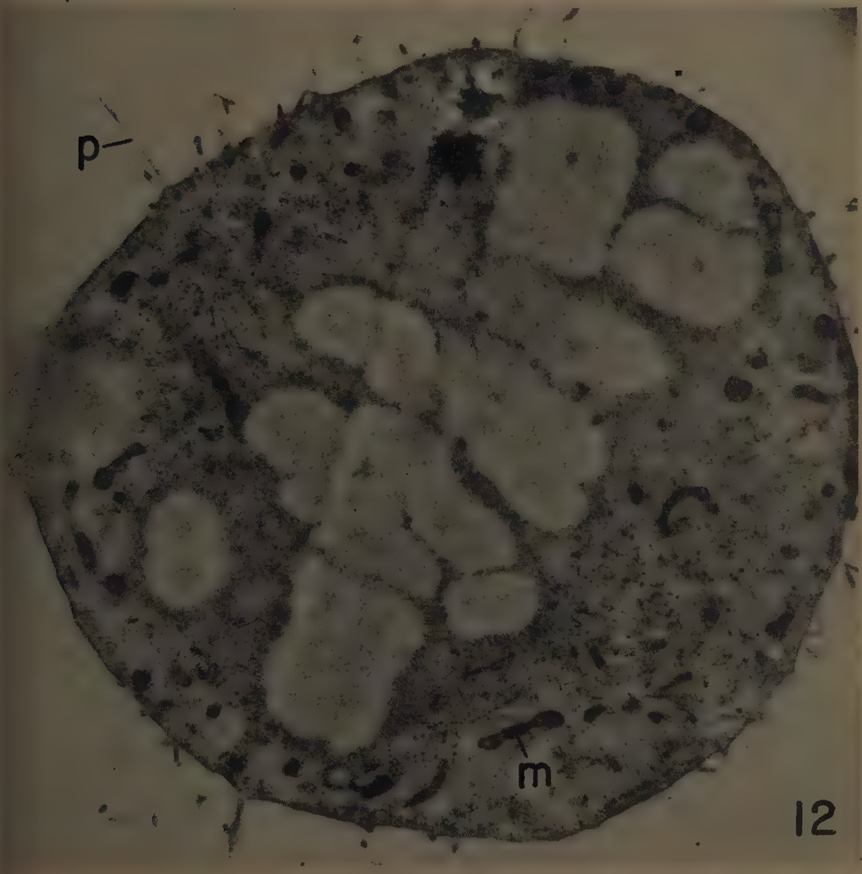


FIGURE 12. Low-magnification view of a section through a metaphase tumor cell that had been stained with 1 per cent PTA following fixation (see section on Method). Chromosome material has apparently disappeared, leaving areas of minimal electron density in their place. Cytoplasmic pseudopods (p) and mitochondria (m) are indicated. ×9,750.

denser. A striking change is seen in the chromosomes when cells are staine with 1 per cent phosphotungstic acid at pH 5.4 following the usual osmium fixation. This procedure had been found useful in our studies of skin ultrastructure, because it enhanced the contrast of membranes and filaments without otherwise altering normal cell components (Selby, 1955b). After phosphotungstic acid staining, mitotic cells of the Ehrlich ascites carcinoma appear, a in FIGURE 12. The chromosomes of this metaphase figure are completely dissolved out of the cell. Chromosomes are also dissolved out of the cells i prophase before disappearance of the nuclear membrane. The unfixed nuclei acid and the protein component of the chromosomes are evidently remove from the cells by the conditions of phosphotungstic acid staining to which the are subjected.

In FIGURE 13 a resting cell, also stained with phosphotungstic acid, is illus-

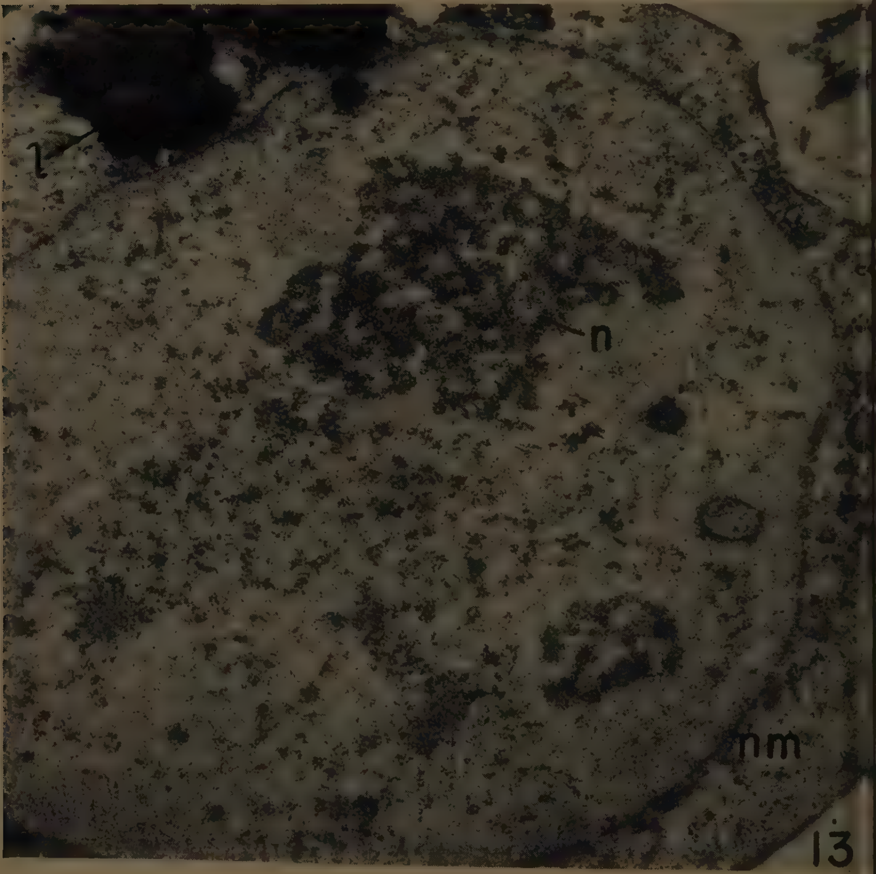


FIGURE 13. Section through the nucleus of an interphase cell in the same specimen in which the cell of FIGUR 12 was observed. Areas of density, usually associated with the nucleolus (n), are distributed throughout th nucleus as a result of the PTA staining. Large lipid droplets (l) and the nuclear membrane (nm) are indicated ×13,500.

rated in order to show the relative lack of effect of the staining procedure on ell components other than chromosomes. There is a change in the nucleus, owever, as additional dense material is evident in it, suggesting a dispersion f strands of nucleolar material throughout the nucleus as a result of the treatment.

A final word may be added concerning other ascites tumors. Studies with hin sections of a Crocker mouse sarcoma 180 ascites tumor and the Krebs II scites carcinoma, although less extensive than our studies with the Ehrlich scites carcinoma, indicate that these other two tumors have no qualitative ytoplasmic difference from the Ehrlich cells.

Summary

Ascites tumor cells have a number of technical advantages over solid tissue for rigorous cytological studies, as with the electron microscope. These cells possess the same components that have been described in high resolution studies of many other cell types, and they have a singular lack of specific granules or filaments such as occur in highly differentiated cells of various types. The sparse development of endoplasmic reticulum suggests that it is almost inactive. The submicroscopic particulates mostly occur freely in the cytoplasm although some are associated with the reticulum when it is present. The projections of the cell membrane are nonspecific in that they may be found along all free cell surfaces and even within vacuoles of the cells. The membranes and vesicles of the cell center are similar to those that have been associated with the Golgi apparatus, agranular reticulum, and centrosphere in other cells. Mitochondria show no qualitative differences from those of normal cells, nor do the submicroscopic particulates. The cell component perhaps least understood is the osmiophilic granule in its surrounding vesicle, which is often seen in the central region and less often in the cell periphery. All the other cell components are known to be present in many or all types of normal cells. Whether this granule is a normal component of the cell center or bears any relationship to neoplasia must await further study. The conclusion may be drawn that no cell components within the resolution limits of this study show qualitative differences from those of normal cells, with certain possible exceptions that demand further study.

During mitosis, cytoplasmic components change position: mitochondria are associated with chromosomes in late prophase but, together with the endoplasmic reticulum, are marginated throughout the rest of division. The vesicular component of the cell center is distributed among chromosomes at metaphase and lines up perpendicular to the division furrows in telophase. Submicroscopic cytoplasmic filaments, which may be present but dispersed throughout interphase, become grouped into bundles resembling chromosomal fibers at anaphase. As cells deteriorate and age, mitochondrial internal structure becomes disoriented and swollen and, eventually, produces small lipidlike droplets, the number of large lipid droplets and vacuoles increases, and the number of submicroscopic particulates decreases.

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Discussion of the Paper

Doctor A. J. Dalton (*National Cancer Institute, National Institutes of Health, Bethesda, Md.*): Using a chrome-osmium fixative[1], we have been studying the ultrastructure of a series of tumor cell types grown in the peritoneal fluid of the mouse, among them S-37. Since a different fixative and different

FIGURE 1. Electron micrograph of part of the cytoplasm of an interkinetic cell of tumor S-37 grown in peritoneal fluid. Many of the small (150 Å) granules of Palade are free in the cytoplasm. Chrome-osmic fixation (pH 7.2). Approximately ×34,000.

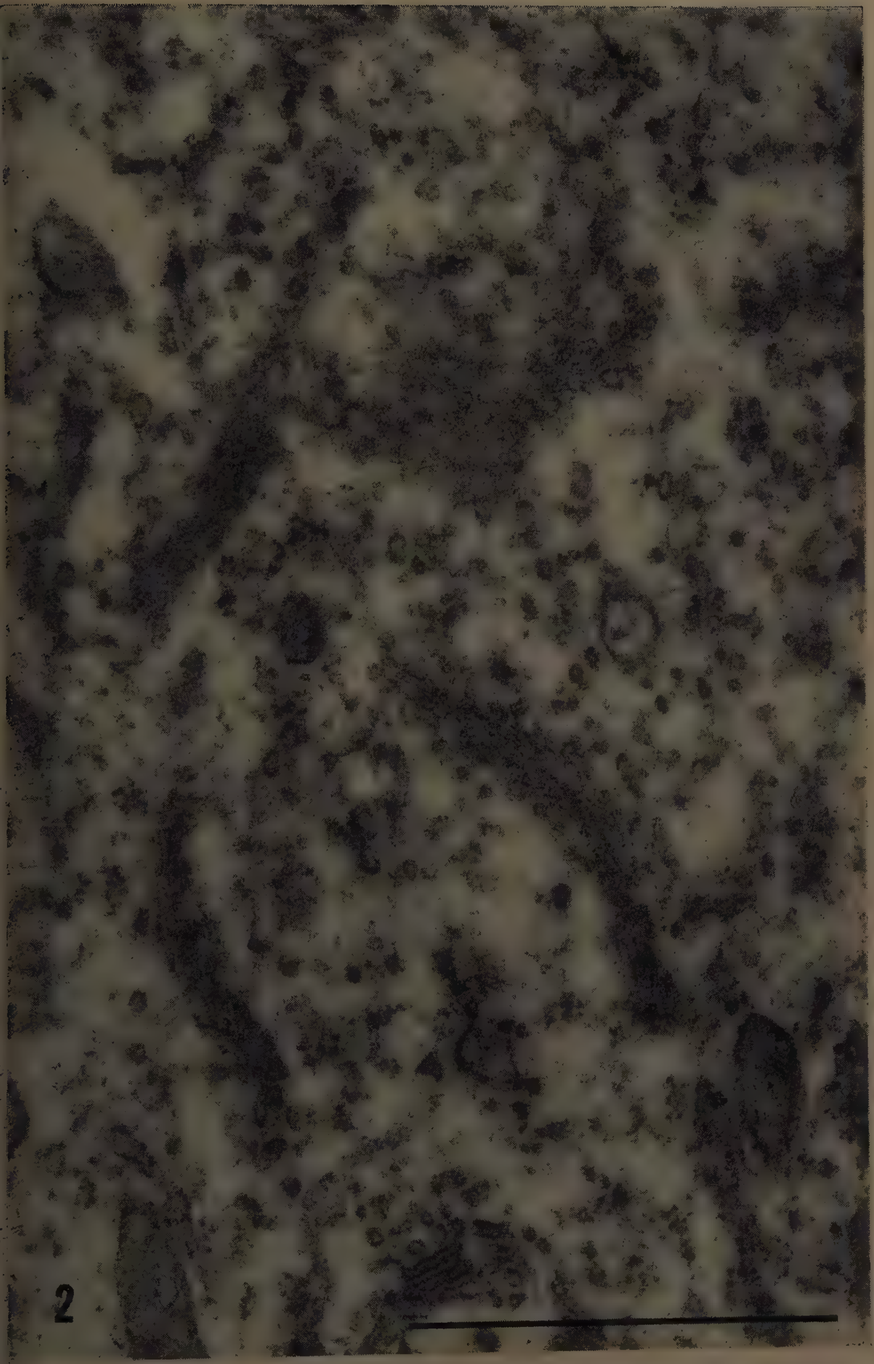

FIGURE 2. Electron micrograph of the Golgi zone of an S-37 tumor cell grown in peritoneal fluid. The Golgi complex consists of groups of double membranes, representing profiles of flattened sacs cut in vertical section and clusters of small granules approximately 400 Å in diameter possessing an outer margin of high electron scattering power and an electron lucent center. Chrome-osmic fixation (pH 7.2). Approximately ×57,000.

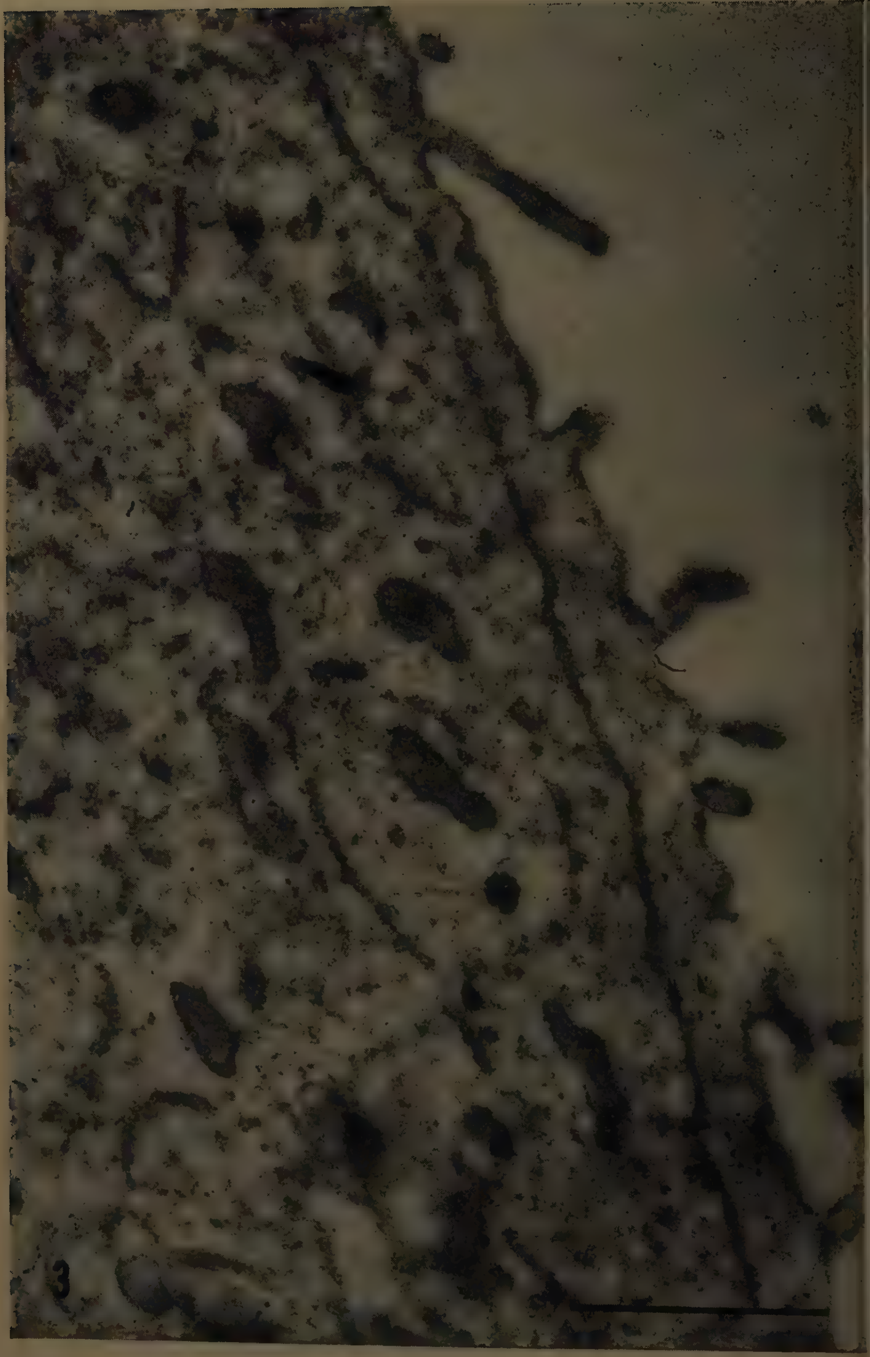

FIGURE 3. Electron micrograph of part of the cytoplasm of a cell of tumor S-37 in division showing cell processes and margination of what appear to be ergastoplasmic membranes. The small (150 Å) granules of Palade show no affinity for these membranes. Chrome-osmic fixation (pH 7.2). Approximately ×34,000.

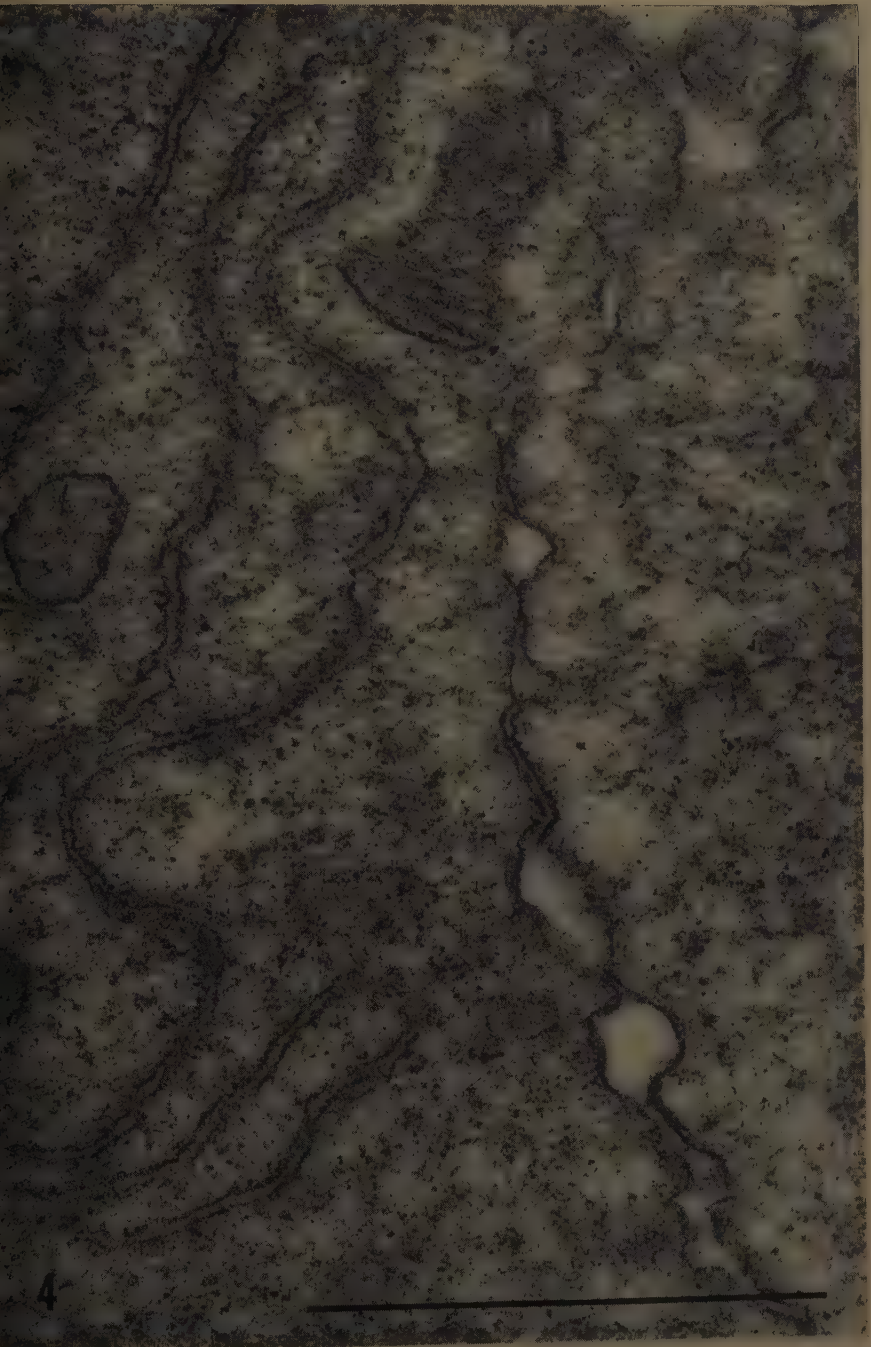

FIGURE 4. Electron micrograph showing contact between two S-37 tumor cells grown subcutaneously. The cell processes characteristic of cells grown in peritoneal fluid are not in evidence. Chrome-osmic fixation (pH 7.2). Approximately ×69,000.

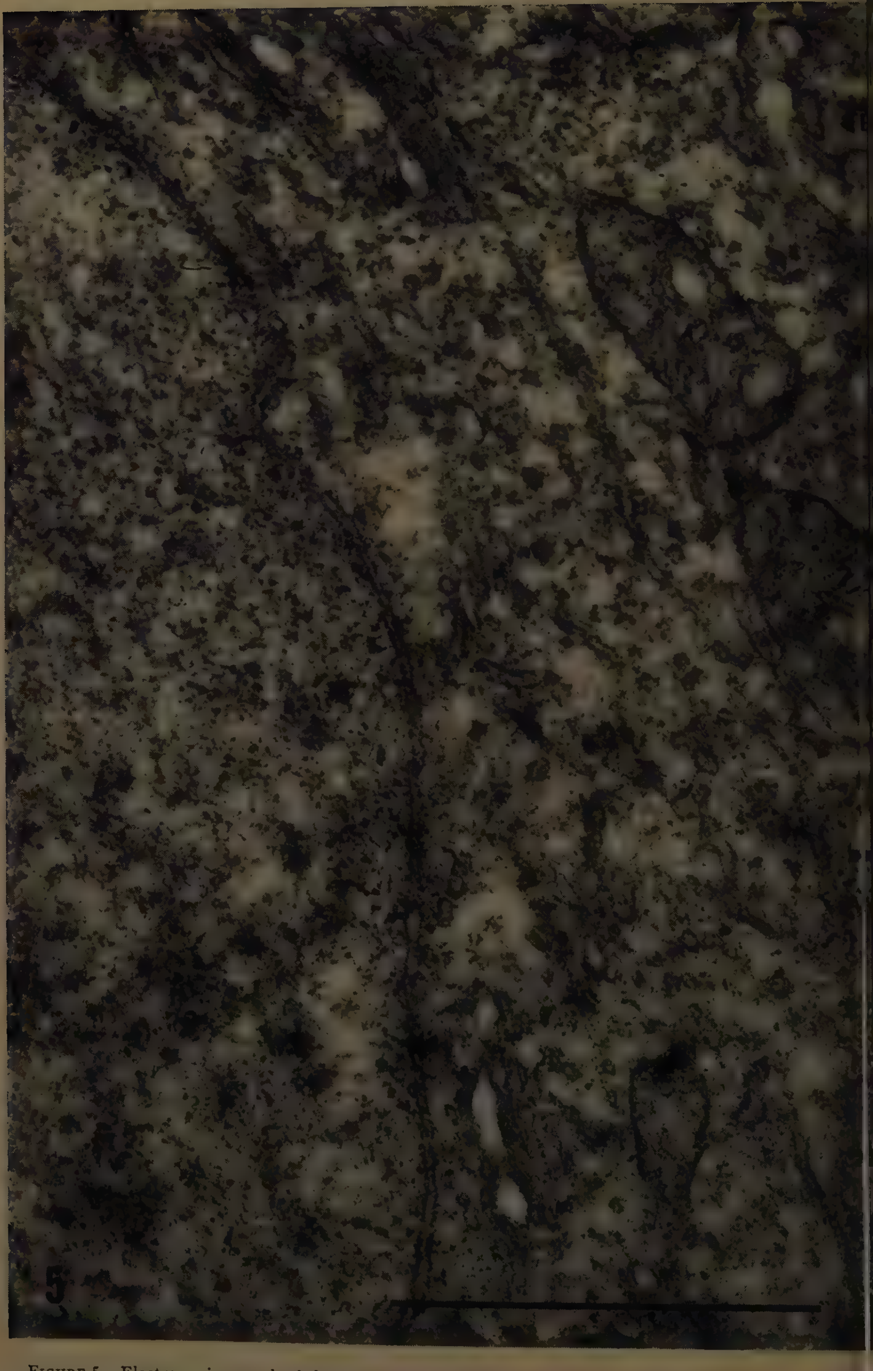

FIGURE 5. Electron micrograph of the Golgi zone of an S-37 tumor cell grown subcutaneously. The Golg complex consists of groups of double membranes, clusters of small (400 Å) granules plus discrete vacuoles marginated by the double membranes. Chrome-osmic fixation (pH 7.2). Approximately ×57,000.

tumors were involved, a comparison of our results with those of Selby *et al.* should be of interest. In addition, I should like to compare the ultrastructure of S-37 cells grown intraperitoneally with those grown subcutaneously.

With but a few minor exceptions, our results agree with those of Selby *et al.* One difference is that we could find little or no effect of centrifuging cells before fixation. Both nuclear and cytoplasmic detail appeared to be preserved well whether the cells were fixed immediately after their removal from the peritoneal cavity or fixed after centrifugation. S-37 tumor cells could well be less sensitive to centrifugation than Ehrlich tumor cells. Cytoplasmic components of S-37 cells are similar to those described for Ehrlich tumor cells, except that the small 150 Å. granules first described by Palade[2] are usually, but not always, associated with the membranes of the ergastoplasm (FIGURE 1). The structures described by Selby *et al.* as part of the Golgi complex have been interpreted by us to be groups of paired membranes, which are actually profiles of flattened sacs in vertical section (FIGURE 2). Serial sections tend to support this view. One other difference is that the small (0.1 μ), dense granules contained within vesicles described by Selby *et al.* have not been identified in the cytoplasm of S-37 cells. The margination of ergastoplasmic membranes described by them, however, is particularly marked during mitosis of S-37 cells, and equally noticeable is the absence of the small (150 Å.) granules usually associated with these membranes (FIGURE 3).

A comparison of S-37 cells from peritoneal fluid with those grown subcutaneously reveal two distinct differences. First, the slender cell processes characteristic of the cells from peritoneal fluid are lacking for the most part on cells grown subcutaneously (FIGURE 4). Second, while the smooth double membranes and small (400 Å.) granules characteristic of the Golgi complex of S-37 cells from peritoneal fluid are also present in cells grown subcutaneously, these latter cells frequently possess small discrete vacuoles as part of the Golgi complex (FIGURE 5).

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CHROMOSOMES IN CANCER TISSUE

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The cancer literature contains a great number of papers providing information about chromosomes in cancer tissue. Very few of these investigation however, have chromosomes as their main issue, or are written by cytologist In the majority of papers, the information about chromosomes is given mor or less in passing, as side-products of pathological, chemotherapeutic, or othe investigations. Data on cancer chromosomes have been gradually accumulating, however, and it was realized early that chromosomes in cancer tissue behaved somewhat differently from those of normal tissue: the chromosoma mechanism functioned less accurately, mitotic deviations of all kinds bein frequent.

Ever since the end of the last century, etiological connections have been suggested between chromosomal aberrations and the origin of malignancy. Name such as von Hansemann and Boveri are associated with this idea. Winge[1,] is the first modern cytogeneticist to stress the tumor tissue as a geneticall heterogeneous cell population and to visualize the origin and growth of tumor as a result of selective processes among the constituents of this population He based his ideas on careful and extensive chromosome studies in plant an animal tumors. The following quotation out of Winge's 1930 paper[2] (p. 727 illustrates his view of the origin and growth of tumors: "Ich betrachte de malignen Tumor als bestehend aus Abkömmlingszellen von einer oder mehrere Zellen, die durch eine lokale Reizung einen abnormen Chromosomenbestan bekommen haben, in dem besonders häufig ein Verlust verschiedener Chromosomen und eventuell Verdoppelungen anderer Chromosomen eingetreten ist was eine vergrösserte Wachstumsintensität und im übrigen einen gestöhrte Metabolismus mit sich führen kann." This quotation shows that Winge wa well aware of the stemline concept. Through genetic variation in the tumor manifested as asymmetric or multipolar mitoses, the basis for an increase i virulence is available: "Es geschieht während des Vordringens des maligne Gewebes eine Selektion unter den aberranten Zellen" (p. 728). Winge considers chromosome doublings as especially significant for the development o malignancy. In his analysis of tar carcinomas of mice, he observed that th most malignant neoplasms usually had hypotetraploid chromosome numbers while precancerous stages still maintained near-diploid numbers.

Winge's view of the cancer tissue as genetically a mosaic, in which the mos viable cell lineages are selected to propagate the tumor, is still largely valid On the other hand, it does not seem to follow necessarily that the original primary induction of malignancy is caused by the same factors as those responsible for changes in the established tumors, as evidently assumed by Winge It is one of the most urgent tasks for chromosome research in cancer to focu on the early stages of cancer growth, or even on precancerous stages.

The discovery during recent years that ascites tumors permit a much mor detailed chromosome analysis than solid tumors has given new zest to the chromosome study of tumors. A great number of new data has been gathered during the last five years through the work of Makino,[3, 4] Makino and Kanô,[5] Bayreuther,[6] Levan and Hauschka,[7, 8] Tjio and Levan,[9] and others.

In the present report, I shall survey briefly some recent developments in this line of research. I shall discuss particularly observations of chromosome differences between tumors and their original host species, and tentative conclusions as to the origin of such differences. It should be observed that our knowledge of chromosomes in tumors is essentially limited to ascites tumors. Results from ascites tumors may not be directly generalized, since we know that important classes of tumors have eluded, so far, all attempts to transform them into ascites.

Although I shall take into account work by other investigators, the present paper is based mainly on investigations within my personal range of experience. My work with mouse ascites chromosomes started in 1951 at the Institute for Cancer Research in Philadelphia and continued there during 1952 and 1954 with support from the United States Public Health Service, Bethesda, Md. It was done in continuous cooperation with Doctor T. S. Hauschka. I shall report, further on, the work with mouse, rat, and human ascites done at the Cancer Chromosome Laboratory, Lund, Sweden, financed by the Swedish Cancer Society. Results were obtained in cooperation with Mrs. E. Melander and Doctor J. H. Tjio. The Lund laboratory has the advantage of cooperation with certain medical institutions of the Lund University and the Karolinska Institute, Stockholm (Doctors C. G. Ahlström and G. Klein).

Numerical Conditions

It is now well known that the chromosome numbers of tumors show greater imbalance than those of normal tissue. They exhibit different kinds of variation caused by different cytological mechanisms. Small fluctuations around a modal chromosome number are caused by anaphasic disturbances of the nondisjunction type. Occurrence of chromosome doublings is induced in various ways (FIGURE 1), as by endomitosis, endoreduplication,[8] c-mitosis,[10] or nuclear fusions. More unpredictable changes are caused by the functioning of multipolar spindles.

The most common chromosome number of a tumor, the modal number, is representative of the cell lineages mainly propagating the tumor, *i.e.*, the stemline number (s). Each tumor generally has a secondary mode in the region of the double stemline number ($2s$) and, sometimes, in addition, a small tertiary mode at $4s$. It was found early that the stemline number may be different from the somatic number of the host species ($2n$). Thus, in a group of mouse tumors studied by Levan and Hauschka,[8] some of the tumors had s near $2n$ of the mouse, some had s near the double somatic number, that is the tetraploid number ($4n$). Still other tumors took an intermediate position.

TABLE 1 includes a collection of mouse, rat, and human ascites tumors arranged in relation to the chromosome number of their host species. Concerning the tumors tabulated, the following references should be made: S3A is

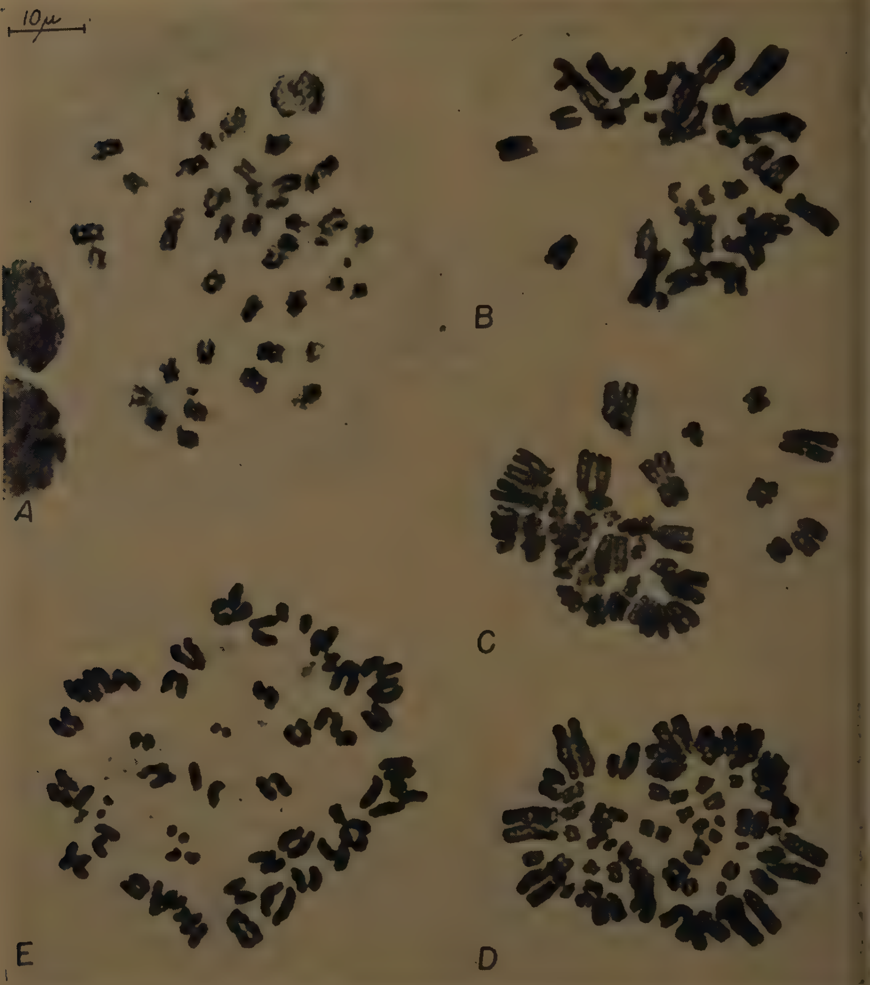

FIGURE 1. Instances of cytologic mechanisms for the induction of chromosome doublings. A: endomitosis, B–D endoreduplication, E: late prophase following c-mitosis. A, E: hyperdiploid Ehrlich mouse carcinoma, B–D hypodiploid Yoshida rat sarcoma. ×1000 (Tjio and Levan, 1954, and unpublished).

quoted from E. Klein,[11] the Takizawa mouse tumor from Yosida.[12] The rat tumors have been reported by various Japanese workers.[3, 5, 13, 14, 15] Rat tumors marked K-M belong to a group of sarcomas experimentally induced by Doctor S. Kullander and analyzed at our laboratory by Doctors E. and Y. Melander (still unpublished). The human tumors K–M were studied by the same persons, while the tumors A–L I and II were studied by Doctor U. Ising and myself. One of the human tumors, finally, is a melanoma ascites quoted from Hsu.[16]

The main interest of TABLE 1 is centered on the fact that tumor development

TABLE 1

SOME ASCITES TUMORS OF THE MOUSE, RAT, AND MAN

IN EACH CASE THE STEMLINE NUMBER IS RECORDED AND RELATED TO THE NEAREST MULTIPLE OF THE HAPLOID NUMBER OF THE HOST

	$1.5x$ $2x$	$2x$ $2.5x$	$2.5x$ $3x$	$3x$ $3.5x$	$3.5x$ $4x$	$4x$ $4.5x$
Mouse $2x = 40$		6C3HED 40 DBA 42 A 2 44 TA3 40, 41 Ehrlich 45, 46 S37^1 40		TA3 68	TA3 70 MC1M 70 Krebs 2 74, 79 13 Krebs 2 clones 71–75 Ehrlich 76, 78 7 Ehrlich clones 73–78 S37^1 78 S3A 72 Takizawa 76	Krebs 2 80 Ehrlich 80, 83 Ehrlich clone 82, 85 MC1A 80 15091a 80 A 1 80
Rat $2x = 42$	Yoshida 40 MTK I 40 MTK II 40 Hirosaki 40 7 Hirosaki sublines 37–39 Hepatoma 40 K–M VII 38	K–M IX^2 42 K–M I^2 44			K–M VI 72 K–M III 76 K–M V 82	Takeda 84 K–M IX^2 84 K–M I^2 88 K–M II 88
Man $2x = 48$		Hsu, Melanoma 48 K–M XI 48–50 K–M XIII^1 50 K–M X 58 K–M XII 58 K–M I 58, 63 (bimodal)	A–L II 64	A–L I 74	K–M II 94–96	K–M XIII^1 100

1. Two modes present simultaneously, the near-tetraploid mode predominating.
2. The $4x$ stemline was predominant in the original ascites tumor, a shift to a $2x$ stemline occurring during later transplant generations.

in mouse, rat, and man involves similar chromosomal changes. In all of them, aneuploid stemline numbers predominate, located mainly in the vicinity of the diploid and the tetraploid chromosome numbers of the host species, and rarely in between these regions. How much significance should be attached to the minor differences of stemline number distributions indicated between the three species must remain an open question until more data have been gathered.

The collective survey of stemline numbers of tumors in TABLE 1 strongly suggests that numerical changes do occur with the stemlines during their development. It should be emphasized, however, that tumors are generally characterized by a very pronounced constancy, allowing them to keep their cytologic and physiologic characteristics during prolonged serial transfer. Even drastic applications of colchicine or nitrogen mustard, which are seen actually to interfere with close to 100 per cent of the cells, usually fail to induce permanent changes. After the end of the treatment, the tumors will return to normal conditions. The interaction between variability-promoting factors (as mitotic disturbances) and factors counteracting variation (differential viability) will maintain the population at an equilibrium. The stability of the stemline, nevertheless, is not an absolute one. Under certain conditions, shifts will occur in the properties of the tumors, and such shifts will sometimes be demonstrable as visible changes of the stemline chromosome complement. In other words, the functioning stemline may be overgrown by some of the side lines.

Several cases of very pronounced changes in stemline chromosome number have been reported. Thus, the TA3 adenocarcinoma of the mouse has developed near-tetraploid lines in addition to the near-diploid lines[17] and the S3A mouse mammary carcinoma, originating as a solid tumor with 40 chromosomes, spontaneously changed into an ascites with $s = 72$.[11]

Somewhat more difficult to demonstrate are small adjustments in stemline number. That such occur, though, has been established in several instances, a few of which will be briefly mentioned.

Two instances of gradual chromosome number adjustment were observed by Hauschka[17] and Hauschka and Levan (in press) in the near-tetraploid mouse carcinomas Ehrlich and Krebs 2. Thanks to their viability in the frozen tumor bank, samples of these tumors could be simultaneously reinvestigated from different times of their entire transplantation history at Doctor Hauschka's laboratory where they were always propagated in Swiss ♀ mice. As seen in FIGURE 2, both tumors have gone through a decrease in stemline number from early transplant generations (in 1950) to generation 200 (in 1954). Both tumors started out with about 80 chromosomes, both changed to a hypotetraploid level. This happened, as far as known, only with the Swiss-propagated lines of these tumors. In Stockholm and Lund, where they are carried in less uniform genotypes, they still have *s* at about 80. In Philadelphia, we happened to witness the slow adaptation of these tumors to the environment imposed on them there, *i.e.*, mainly the host genotype. In Buffalo (Doctor Hauschka's present location), the Ehrlich and Krebs 2 ascites are maintaining their hypotetraploid character in Swiss mice.

Another difference of this kind was demonstrated by Tjio and Levan[9] between different lines of the hyperdiploid Ehrlich carcinoma. One line had a narrow distribution of chromosome numbers with $s = 46$, while another line had a wider distribution with $s = 45$. This difference was maintained for the entire period of about one year, during which the tumors were continuously controlled at our laboratory.

Similar chromosome number adjustments were recently observed at our laboratory for a human tumor, K–M I. This tumor, an ovarian cystocarcinoma ascites, was sampled twice with an interval of 10 days between. At both samplings, the chromosome numbers formed a bimodal distribution, indicating the presence of two stemlines (at 58 and 63). Between the two samplings, however, a significant shift in chromosome number distribution had

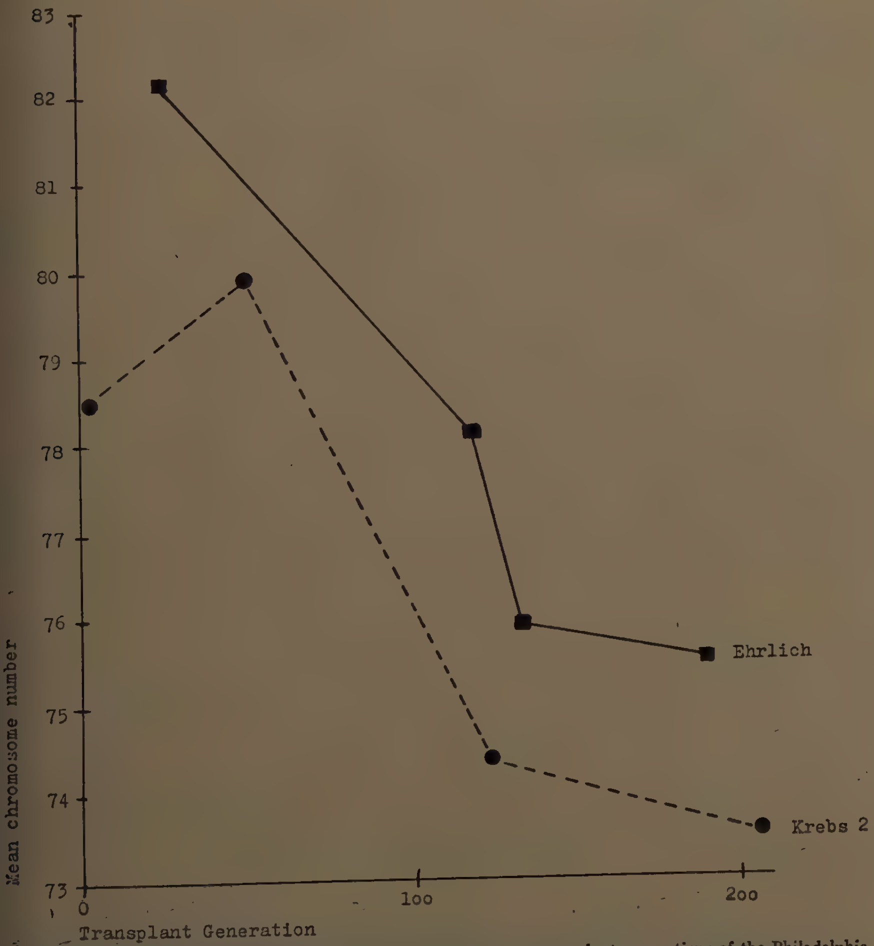

FIGURE 2. Graph of stemline chromosome numbers during 200 transplant generations of the Philadelphia lines of the hypotetraploid Ehrlich and Krebs 2 mouse carcinomas (Hauschka and Levan, unpublished).

TABLE 2

DISTRIBUTION OF MEAN CHROMOSOME NUMBERS IN CLONES AND SUBLINES FROM THE HYPOTETRAPLOID MOUSE ASCITES TUMORS KREBS 2 AND EHRLICH*

Stock tumor	Clones and sublines									Number of cases	Average
	Average chromosome number										
	71	72	73	74	75	76	77	78 — 84	85		
Krebs 2 (74)	2	1	5	3	2	—	—	—	—	13	73.6
Ehrlich (78)	—	—	1	2	1	2	1	—	1	8	76.8
Total	2	1	6	5	3	2	1	—	1	21	74.8

* Hauschka and Levan, unpublished.

taken place in favor of the 63 chromosome stemline (E. Hansen-Melander, Kullander and Y. Melander, in press).

An excellent way of demonstrating the numerical heterogeneity of the tumor cell populations is the analysis of different single cell clones derived from a stock tumor. I had the advantage of studying, together with Doctor Hauschka, the chromosomes of the clones isolated by him from hypotetraploid Ehrlich and Krebs 2.[17] These clones are characterized not only by their individual physiological properties but also by individual stemline chromosome numbers. In TABLE 2, the stemline numbers of about 20 clones are recorded together with the stemline numbers of the mother tumors at the time when the clones were picked out. Evidently, cells that in the stock tumor have already declined from the stemline and would probably be of minor importance to the tumor may develop into tumors of their own, if isolated.

In summary, it may be stated that chromosome number changes play an important role in the development of tumors. The changes may be of the type chromosome doublings or small adjustments. Cytologic mechanisms for these processes are constantly available. Permanent shifts in stemline chromosome number will require, however, a change in the environment of the tumor and/or an intrachromosomal rebalancing of the genome. This latter type of genotypic changes will be the subject of the next section of this paper.

Structural Conditions

That mechanisms for intrachromosomal alterations are at work in the tumors is evident from almost all, even the most superficial, studies on mitosis in cancer tissue. A leading feature in all descriptions of chromosome disturbances in tumors has been the so-called unclean anaphase separation, *i.e.*, bridge formations of various kinds. The important technical progress connected with the exploitation of ascites tumors for chromosome study now makes it easy to study these disturbances at anaphase and to analyze at metaphase the configurations underlying the anaphase disturbances. FIGURE 3 shows instances of structural changes; *viz.*, dicentric chromosomes at metaphase and a criss-cross bridge at anaphase, the latter configuration being the direct consequence of a dicentric metaphase chromosome. Other kinds of easily recog-

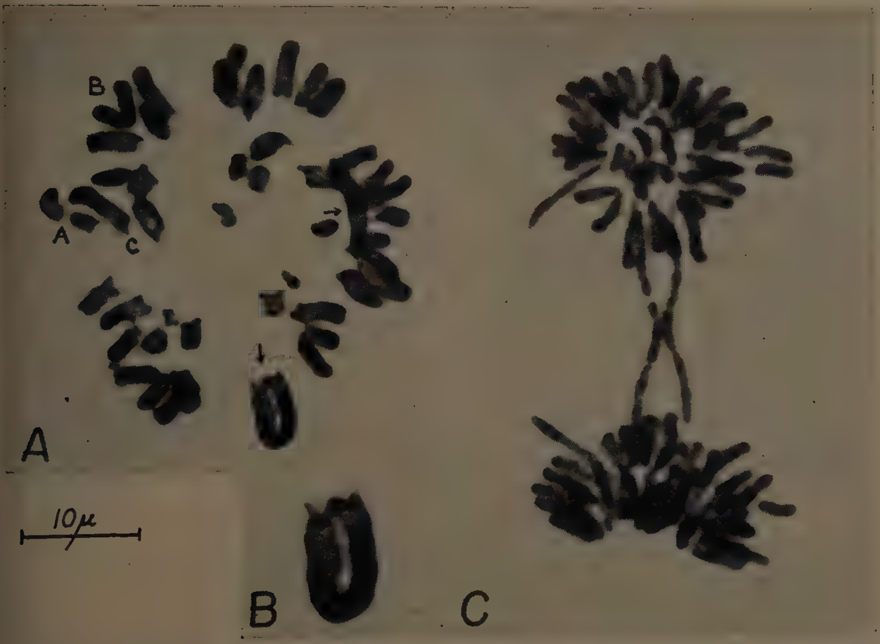

FIGURE 3. Instances of structural chromosome variation. A: metaphase with two dicentric chromosomes (at the arrows), B: detail of one of the dicentrics of A, C: anaphase with criss-cross bridge. Hyperdiploid Ehrlich mouse carcinoma. A, C: ×1500 (Tjio and Levan, 1954).

nized structural changes are pseudochiasmata, ring chromosomes, and centric and acentric fragments.

It is of interest that certain structural abnormalities may become self-propagating and may even enter the stemline of a tumor. Koller[18] describes such a case of dicentrics in a rat carcinoma. In my own experience, two cases of stemlines with a ring chromosome in human tumors have been encountered. In one of these, a pleural transudate from a pulmonary tumor, one large ring chromosome was present in nearly all cells. This ring still persisted at a second sampling of the tumor, four weeks later. The size and the clearness of the ring permitted a close study of its behavior at normal mitosis and after endoreduplication.

The incidence in one cell of many chromosome breaks is not infrequent in untreated tumor material. One instance of such a cell is seen in FIGURE 4. It contains a great number of chromatid reunions in new combinations.

The origin and maintenance of very small chromosomes ("minutes") is often seen. Sometimes their size is extremely tiny, little larger than centromeric bodies. Still, they may have the capacity of behaving normally during mitosis and of entering the stemline. In our study of Ehrlich and Krebs 2, stock tumors and clones (Hauschka and Levan, in press) we followed the behavior of such minutes. In the stock tumors, they were seen only exceptionally, but in the clones (especially in those of Krebs 2) they were surprisingly fre-

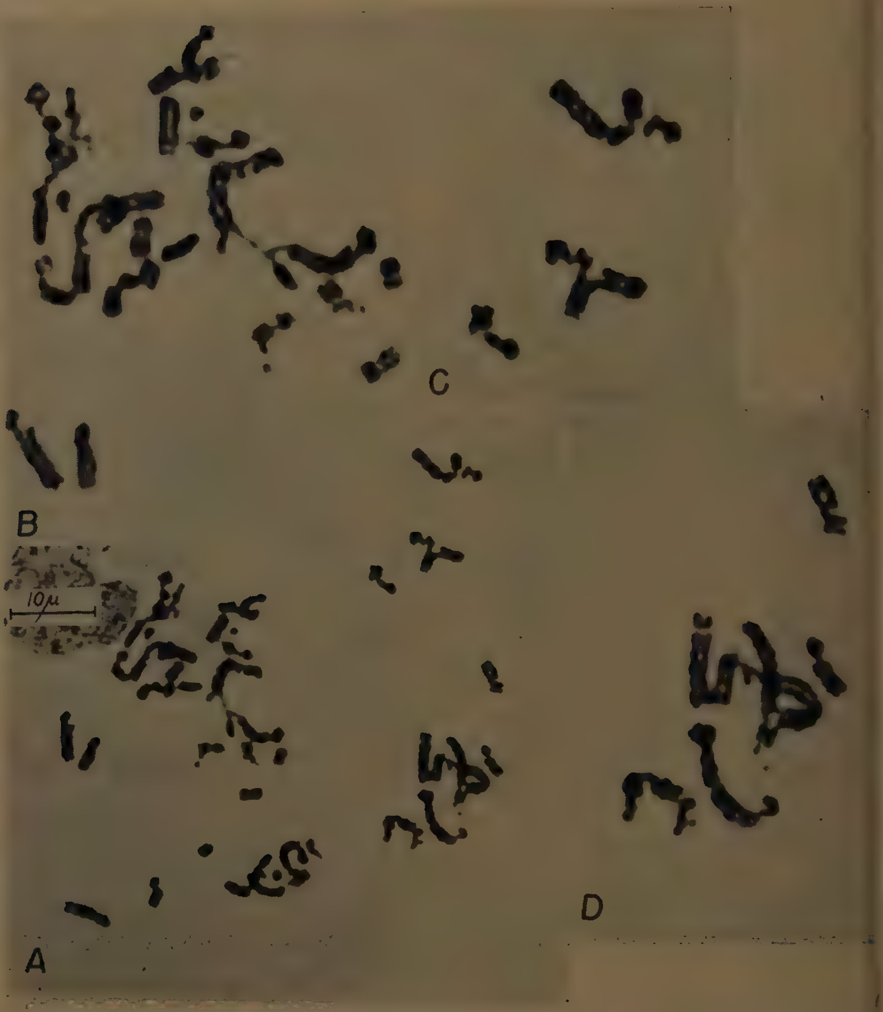

FIGURE 4. A: cell at late prophase, showing many spontaneous structural changes, B–D: details at higher magnification. Yoshida rat sarcoma. A: ×1100 (Tjio and Levan, unpublished).

quent. No less than 7 out of 11 Krebs 2 clones had one or more minutes as regular members of their stemline idiograms. Since the frequency of minutes in the mother tumors was decidedly lower, it seems that the presence of minutes in that material meant a favorable quality for the establishment of a clone.

The dicentrics and the rings certainly handicap normal mitotic function. The fact that they, as well as the minutes, are still maintained in the stemlines of many tumors indicates that they carry indispensible genic material, or else they would have been eliminated.

Structural variation, a few instances of which have now been mentioned, is always available to the tumors. That they make use of this variation for remodeling their idiograms is evident from the many permanent structural differences demonstrated between the stemlines of different tumors and their host idiograms. I need refer here only to the large V chromosome present in the Yoshida sarcoma, not found in the rat idiogram,[3] the A, B, and minute chromosomes of the hyperdiploid Ehrlich, first described by Bayreuther,[6] and to other similar innovations in several other mouse tumor idiograms.[8]

The structural changes so far discussed are all very striking and may be referred to as the *qualitative* or the gross type of changes. I intended, however, to investigate whether, possibly, structural rearrangements of another, less striking kind occur; *viz.*, rearrangements identifiable only by actual measurements of chromosome lengths. Such changes will be referred to as *quantitative* or cryptostructural changes.

The mouse idiogram, consisting of only 40 telocentric elements, is better suited for quantitative analysis than the rat or the human idiograms, which contain 66 and 96 chromosome arms, respectively. Ten spermatogonial metaphases were carefully drawn, each chromosome being outlined with camera lucida in the center of the viewfield at high power. They were, thereafter, measured with a ruler graduated in 0.1 μ. In order to eliminate occasional differences in contraction state, all chromosomal measurements were recalculated to the same average chromosomal length per cell. I thus obtained a standard idiogram for the germ-line chromosomes of the mouse.

Now the same procedure was repeated with the chromosome sets of different tumors. In spite of the high number of chromosomes in some of them, there was no difficulty of finding metaphase plates with all chromosomes in one plane.

In order to make comparisons possible between the different groups of material, thus measured, the values obtained were represented as histograms for the distribution of chromosome lengths. One histogram was drawn for each metaphase plate measured, each length class of the histogram being made 0.3 μ, and the total area of each histogram being made equal, *i.e.*, differences in chromosome number between normal cells and different tumor cells being equaled out. If no structural differences were present between the normal mouse cells and the tumor cells, all histograms thus constructed should be essentially identical all through the entire material.

In all, 40 metaphase plates from different tumors were measured through, representing 5 plates of near-diploid TA3, 5 plates of each of two hypotetraploid lines of TA3, 15 plates of Krebs 2 stock and sublines, 10 plates of hypotetraploid Ehrlich stock and clones. The latter two materials were studied in cooperation with Doctor Hauschka. The details will be published in a joint paper.

FIGURE 5 gives a summary of the entire material, the 50 histograms having been condensed into four average histograms. It is seen from this picture that the normal mouse chromosomes vary in length continuously from about 2 μ to 5 μ, the intermediate length classes being most common.

The only diploid tumor studied (TA3) shows about the same length variation as the normal mouse cells. No great structural rearrangements need therefore

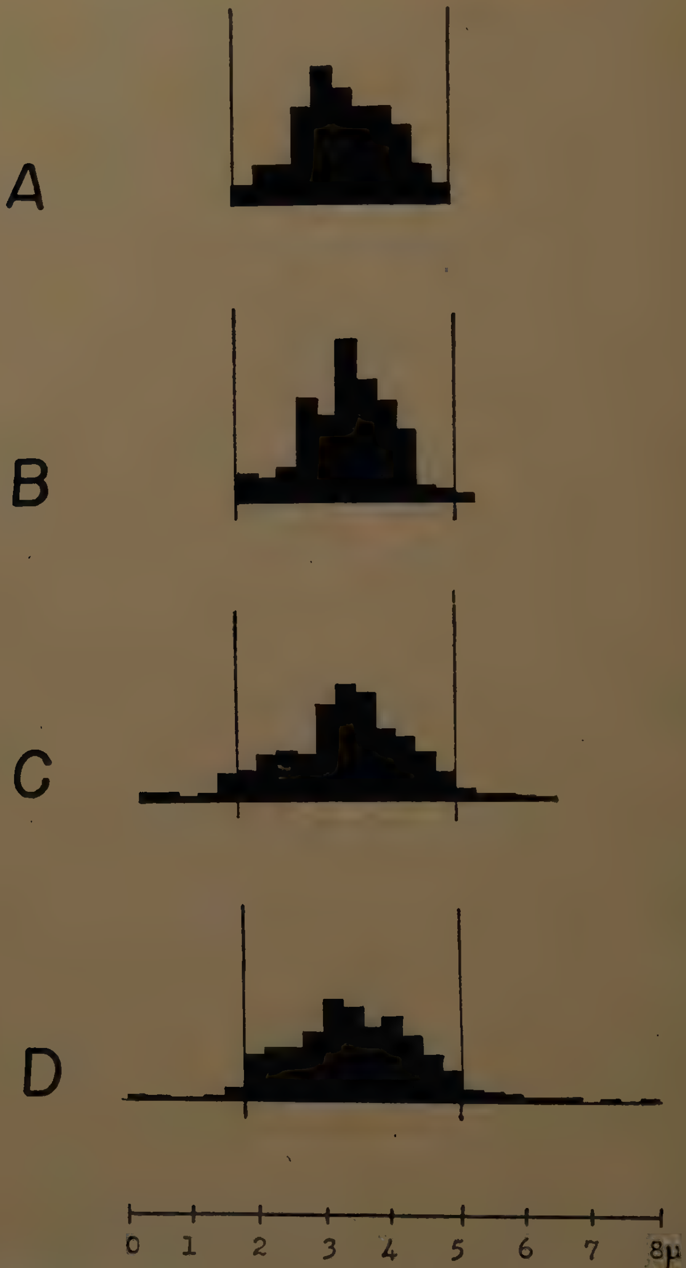

FIGURE 5. Average histograms of the distribution of chromosome lengths for A: normal mouse chromosomes, B: near-diploid TA3 adenocarcinoma, C: near-tetraploid strains of the same tumor, D: near-tetraploid Ehrlich and Krebs 2 carcinomas. A is based on 10 measured metaphase plates, B on five, C on ten, D on 25 plates.

be postulated for the development of this tumor. Preliminary studies of two other diploid tumors, DBA and 6C3HED indicate that conditions are essentially similar in them. It seems that diploid mouse tumors maintain the length relations of the mouse chromosomes, a condition that was already commented upon by Levan and Hauschka[8] from purely qualitative evidence.

It is seen in FIGURE 5 that the situation with the tetraploid tumors is quite different. In no single case of the 35 chromosome plates underlying the two average histograms of this figure did the length variation stay within the limits set by the spermatogonial mitoses. Each single plate had wider spread of the chromosomes towards both sides, while the intermediate types were underrepresented. All chromosome sets of the near-tetraploid tumor cells recorded had greater extremes in chromosome length. This condition is also evident from FIGURE 6, in which averages of the shortest and longest chromosome in each group of material have been represented.

As an instance of the material underlying the above measurements, FIGURE 7 is submitted, showing one spermatogonial idiogram of the mouse together with one idiogram of a near-tetraploid tumor. That the latter type has greater extremes in chromosome length is evident.

It has been established by the evidence now presented that an essential difference exists between the diploid and the tetraploid tumors examined. While the diploid tumors have an idiogram largely of the same morphology as the normal mouse idiogram, the tetraploid tumors have deviating idiograms.

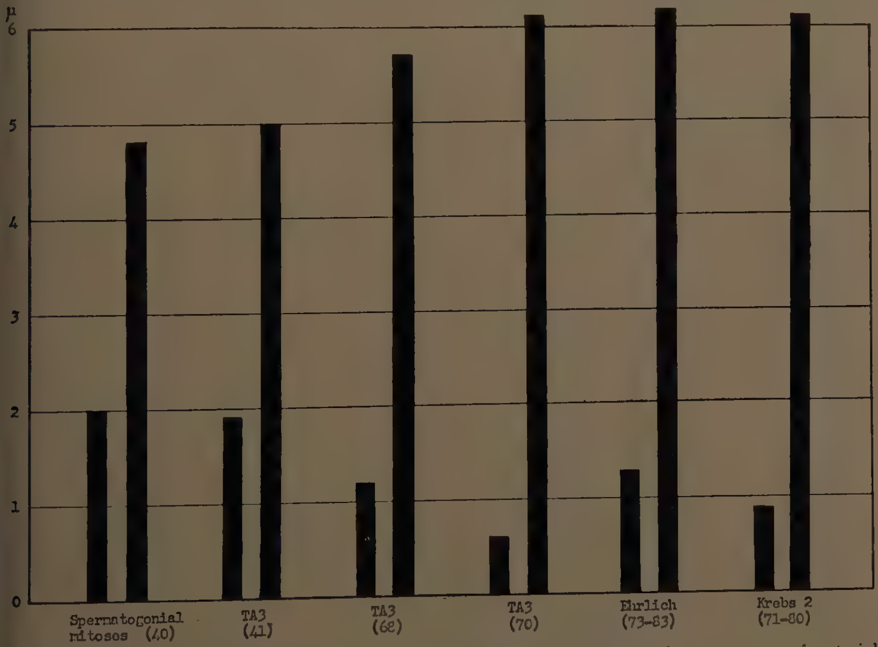

FIGURE 6. Diagram of average length of shortest and longest chromosome in the same groups of material as in FIGURE 5. Chromosome numbers are given below each material.

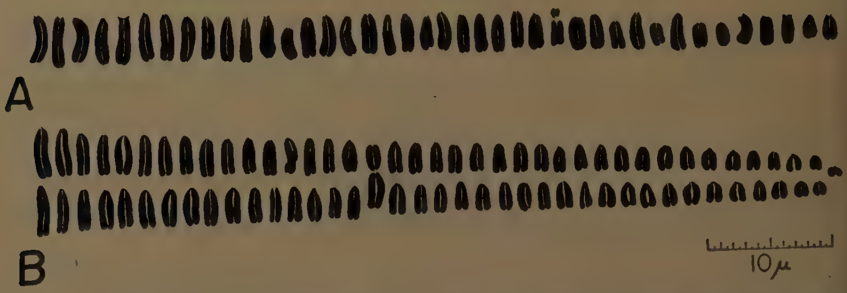

FIGURE 7. Idiograms of A: one spermatogonial metaphase of the mouse, B: one metaphase of the hypotetraploid Ehrlich clone tumor E3. Note greater extremes in chromosome length in the latter metaphase. ×1600 (Hauschka and Levan, unpublished).

The latter contain greater extremes in chromosome length and must have undergone structural changes during their development.

It is easy to visualize the nature of these structural changes. Interchromosomal translocations may lead to recognizable types of new chromosomes, if the chromosome breaks preceding the translocations are located in different regions of the two chromosomes involved (see left part of FIGURE 8). More symmetric translocations will not be identifiable (right part of FIGURE 8).

Evidently, the diploid tumors have undergone no translocations of the more asymmetric type, or cells with such translocations have failed to survive. In the tetraploid tumors, on the other hand, cells with asymmetric translocations have persisted and have even been introduced into the stemlines. Since there is no reason to assume that highly asymmetric breaks should participate in translocations more readily than the more symmetric breaks, it seems reasonable that, for each visible translocation, many others occur that elude observation. Since even quite extreme rearrangements have been shown to be viable in tetraploid tumors, while no rearrangements at all were identified in the diploids, a very significant difference stands out between diploid and tetraploid tumors: *structural remodeling of the chromosome set has played a far more important role in the development of the tetraploid tumor idiograms than in the diploids.*

As is well known from many plant materials, tetraploid genotypes may be expected to be more resistant to genotypic changes than diploids. This constitutes the basis for the greater genotypic adaptability of polyploid plants toward extreme environmental conditions. The relative frequency of polyploids, for instance, is higher in regions with extreme climate, as in the arctic flora. In an autotetraploid, each chromosome is buffered by the presence of three other chromosomes of the same kind, so a genic change or a gross chromosomal change that would be deleterious to the diploid may survive in the tetraploid, because it is compensated for in the rest of the genotype. Speaking purely morphologically, in the present case, we may say that the tetraploid tumors may tolerate greater deviations from the optimal chromosomal length of the mouse than the diploid tumors.

Structural changes may be taken as indications of genic variation in general, both in view of direct gene changes at the points of breakage and of position effects superimposed. Therefore, the tetraploid tumors will contain in their

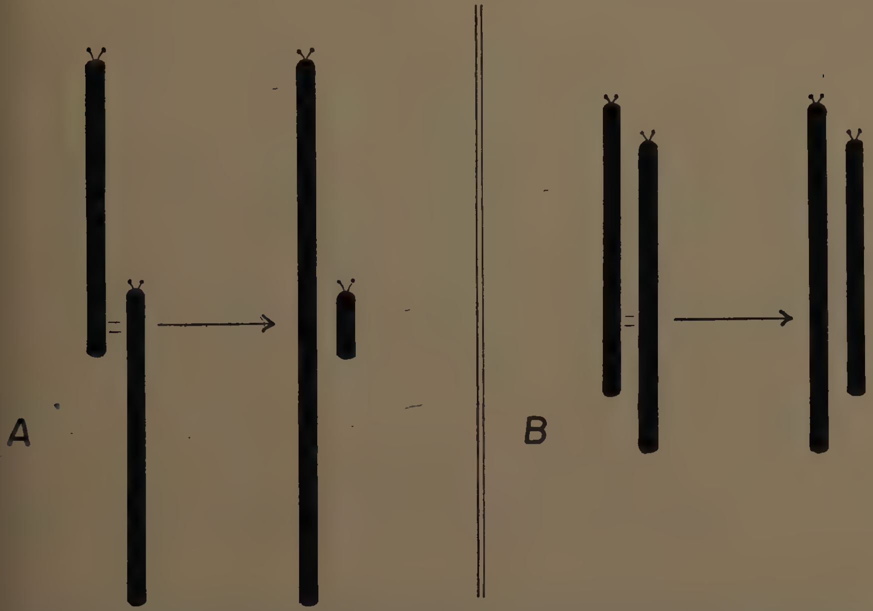

FIGURE 8. Diagram of interchromosomal translocations, A: point of breakage in different regions of the two chromosomes involved; result of translocation visible, B: point of breakage in the same region of the two chromosomes involved; result of translocation not visible.

cell populations a much greater diversity of genotypes. This diversity will form the basis for selection and will appear as a greater genotypic adaptability of the tetraploids, a greater "evolutionary" potential.

We have here the background for the fact that in mouse tumors the most nonspecific and anaplastic ones, those that lend themselves to transformation into ascites, almost consistently show increased chromosome number. This furnishes a tangible cytogenetic background for several of the properties known to be characteristic of tetraploid mouse tumors. For instance, tumors with diploid chromosome number are highly host-specific, while tumors with increased numbers can grow in several host genotypes or are completely nonspecific.[19] The development of this capacity to grow in originally hostile environment is inherent in the greater genotypic variation available on the tetraploid level.

Generally speaking, many qualities of the tetraploid tumors labeled as "improved viability" may be looked upon as expressions of this same condition. Because of their greater supply of diverse genotypes in their cell population, the tetraploid tumors are generally more resistant to unfavorable conditions of all kinds, including radiation, metabolic poisons, temperature extremes, and immunologically resistant environments.

Conclusions

The brief survey of the chromosomal situation in ascites tumors, here presented, has shown that the ascites tumors as a rule have undergone, during their development, numerical and structural chromosome alterations. Only certain lymphomas maintain the original chromosome number and show no traces of structural changes. Because of the manner of growth characteristic of their mother tissue, the lymphomas should be predisposed to life as ascites, making profound genotypic reorganization superfluous.

Tumors of the three animal species here discussed all show structural rearrangements. So far, it has been possible only in the mouse to obtain an idea of the extent to which the detailed structural reorganization may go. Evidence was presented that the so-called cryptostructural variation, hitherto overlooked, plays a very important role in the development of near-tetraploid tumors. The near-tetraploid lines of TA3, for instance, had completely abandoned the idiogram type of diploid TA3 and were indistinguishable chromosomally from any of the old tetraploid idiograms, such as Ehrlich or Krebs 2. It is significant that the structural reorganization in mouse tumors is more extreme on the tetraploid level. Mouse tumors that have succeeded in shifting over their stemline to tetraploidy have therefore every prospect of reaching high viability under adverse conditions.

It is probable that the development of tumors in the mouse is typical of that species only and that the course of development of tumors in other species may be somewhat different. The genotype of each animal species includes certain potentialities for the development of cancer growth, different animals may have different possibilities, and the actual realization of these possibilities depends on cytologic mechanisms, typical for each species.

There no longer seems to be any doubt that the progression of tumors toward increased malignancy is connected with, and caused by, gradual genotypic changes, which render the tumor stemlines more and more efficient. This is essentially the same picture as sketched by Winge 25 years ago, although it is now supported by much more evidence.

The question arises whether it is permissible by homology to extrapolate from this development backward and conclude that the precancerous changes that start off the malignancy are of the same nature. Is it permissible to assume, as von Hansemann, Boveri, and Winge did, that the original step involves genotypic changes as, for instance, the loss of growth-regulating factors with consequent upsetting of the normal immunologic relations between tissue-host organism? The recent chromosomal studies on ascites cancer have added some strength to this old hypothesis by stressing, furthermore, the significance of the genotype in the development of tumors. The decreased constancy of mitosis, found in all tumors, may well constitute the heritage of an original induction of irregularity into the mitotic mechanism, essential for any development of cancer. The present view, in line with Boveri's and Winge's ideas of cancer development, is by no means contradictory to viral or mutagenic hypotheses for the induction of malignancy. It should be remembered that, so far, no crucial evidence is available involving chromosomal mechanisms in the start and the first development of malignancy. The preparatory steps for genetic deviation may well be extra nuclear, *e.g.*, viral infection. Morphologic or genetic evidence for the hypothetical plasmagenes in cancerogenesis, however, is completely lacking.

The behavior of the chromosomes during the subsequent growth of tumors, however, makes them the natural vehicle for the propagation and progression of neoplasia.

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Discussion of the Paper

Doctor George Yerganian (*Children's Cancer Research Foundation, Inc., Boston, Mass.*): Doctor Levan is to be congratulated for his untiring efforts to quantitate karyologic features of tumors containing morphologically similar chromosomes. With the recent collaboration of C. Livingston and B. McKay, I have done related studies with the Chinese or striped-back hamster, *Cricetulus griseus*, a species rarely used until recently because of difficulties with domestication and breeding. Further domestication and the present production of 200 to 300 animals per month has enabled us to explore a wide range of studies for which it is particularly suited. The techniques utilize the very thin and vascular cheek pouch membrane and 11 easily characterized chromosome pairs or types. We are now in the process of studying the distribution and identifi-

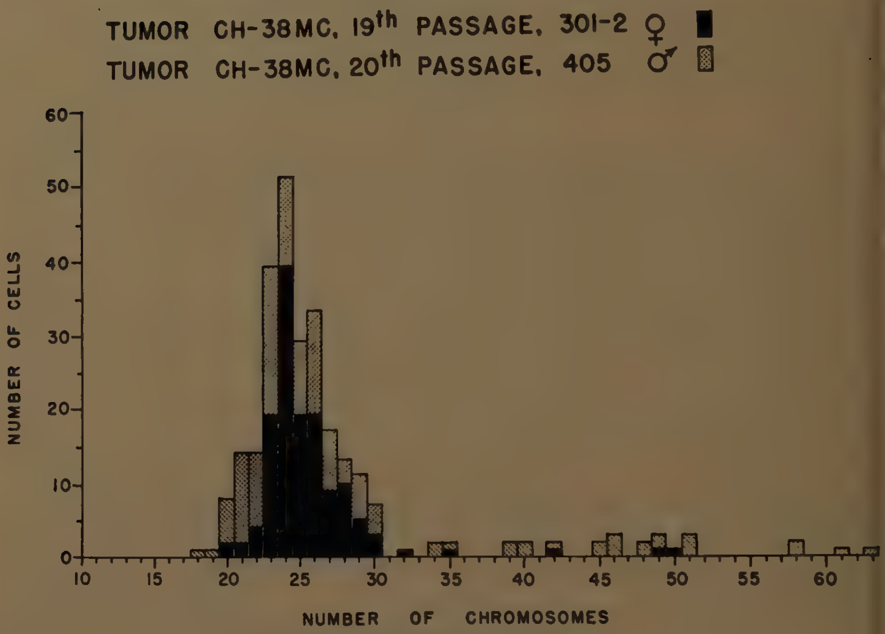

Figure 1. Distribution of chromosomes.

Table 1

Distribution of Chromosome Types

(24 Numbered Cells)

Transplant generation	I	II	III	IV	V	VI	VII*	VIII	IX	X	XI
19th	2	3	3	1		7		2	2	3	1
19th	3	2	3	2		6		2	2	2	2
19th	2	2	4	0		5		2	2	5	2
19th	2	2	2	1		7		2	1	1	6
19th	2	3	4	1		8		1 (?)	2	1	2
19th	2	4	2	2		6		1	3	3	1
20th	3	1	4	1		8		2	2	0	3
20th	6	2	1	2 (?)		7		2	3	1 (?)	0 (?)
21st	3	2	3	0		7		2	2	4	1
24th†	2	3	4	0		7		2	2	2	2
24th	3	2	2	2		7		2	3	1	2
24th	2	3	3	1		7		2	2	2	2
24th	3	2	3	1		8		2	1	2	2
24th	3	2	3	1		7		2	2	2	2
24th	3	3	2 (?)	1 (?)		7		2	4	1	1
24th	2	2	2	3		7		2	1	5	0
24th	2	3	3	2		7		1	1	3 (?)	2 (?)

* Types V, VI, and VII grouped together.
† Colchicine pretreatment of 24th passage.

cation of chromosomes in a methylcholanthrene fibrosarcoma (CH-38MC) grown in peritoneal cavities of selectively bred hosts. Nine of the 11 chromosome types are readily identified. The complement consists of four large-to-medium sized metacentrics or V-shaped chromosomes, three small metacentrics

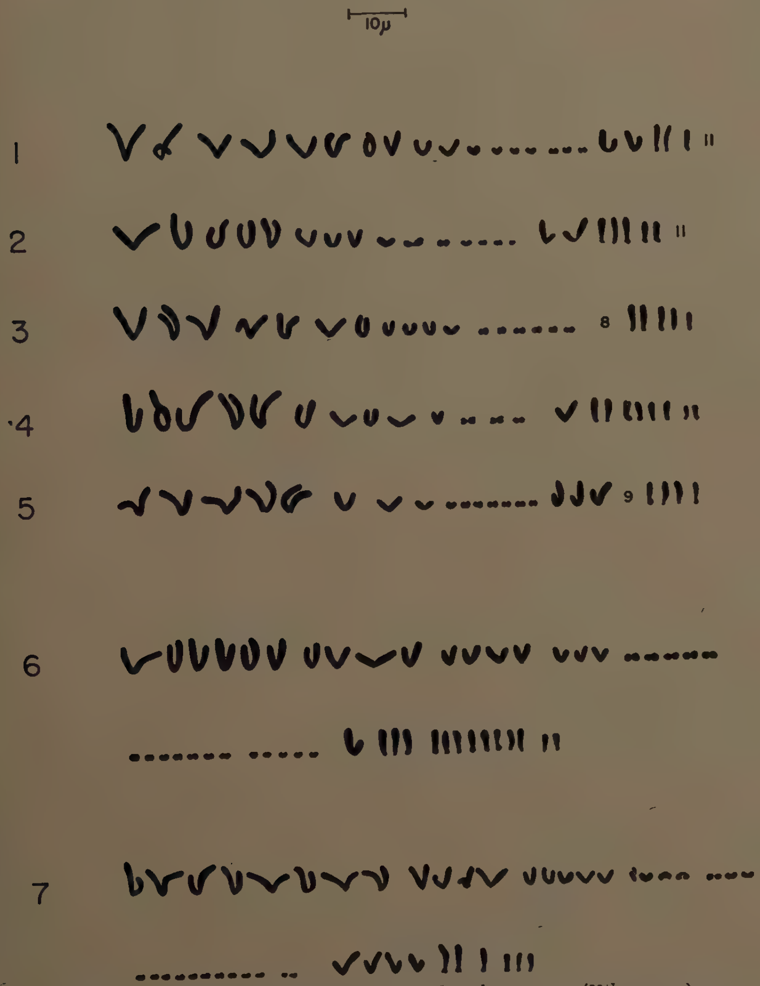

Figure 2. Idiograms of cells having 22, 48, and 46 chromosomes (20th passage).

one large submedial or J-shaped chromosome, and three medium-sized acrocentric or I-shaped chromosomes.

As shown in figure 1, the combined frequency distribution of chromosomes in two sublines revealed a peak modality or stemline of 24 chromosomes along with comparatively high frequencies among cells having 23, 25, and 26 chromosomes. This distribution resembles those described for other tumors, allowing for differences in chromosome numbers of different species.

Idiogrammatic analyses, however, disclosed an extreme randomness in the distribution of the 11 chromosome types in cells having identical chromosome numbers. In other words, specific chromosome types were either missing entirely or duplicated many times in apparently viable cells. Figure 2 provides representative illustrations of this striking feature in cells having 22, 48, and 46 chromosomes. The numbers placed along idiograms signify complete absence of the type in question. In some "diploid" cells, type I was found to be present as often as 5 times. Since types V, VI, and VII were grouped together, we have not differentiated these forms conclusively as yet. Although absence or duplication of specific types were noted in *every* cell analyzed, examples of the distribution among 24 chromosome or "stemline" cells will suffice at this time (TABLE 1).

Failure to demonstrate a definite and replicable chromosome pattern or distribution among stemline cells strongly suggests a random reshuffling of chromosomes as a result of perpetual nondisjunction during bipolar divisions. As in the case of structural variations noted in mouse tumors by Doctor Levan, randomness in chromosome distribution may provide this tumor with a broad genetic potential with which to meet a variety of experimental challenges, or to acquire greater plasticity in the course of future passages.

CYTOLOGICAL VARIABILITY IN HUMAN CARCINOMATOSIS

By P. C. Koller

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The cell population of effusions that are associated with carcinomatosis in man is under conditions greatly different from those that exist in experimentally induced ascites in mice or rats. In the former, the tumor cells are derived not from an inoculum of free cells but either from a primary site or from metastatic adhesions, and they show several interesting characteristics, both in respect to their morphology and behavior, which reveal many of the potentialities of malignant cells not normally observed. These are important properties of malignancy, and they have a great influence on the dynamics of tumors in man.

The present paper describes the chromosomal variability that has been observed in peritoneal and pleural effusions and discusses the cause, the mechanism, and the evolutionary significance of this phenomenon.

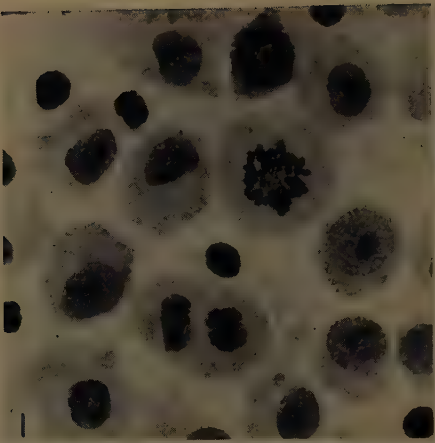
Material and Technique

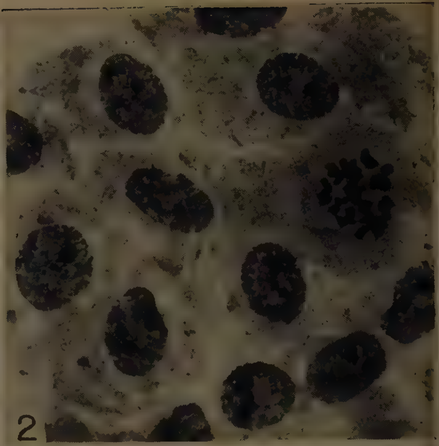
Effusions were obtained from the peritoneal and pleural cavities of 58 selected patients with carcinoma of the ovary, uterus, breast, bronchus, and bladder, and with Hodgkin's disease. The amount of fluid aspirated at each paracentesis varied from 0.5 to 12 liters. Effusions differed in the rate of the retention of fluid. In some, it was very high. The density of cell population in the large volume of fluid was found to be extremely low, and no reliable estimate could be made. Radioisotope studies have shown that an ascites is not a static accumulation of fluid, but that it changes continuously. While significant differences were found in the viscosity of the different effusions, only slight variation was observed in the pH conditions.

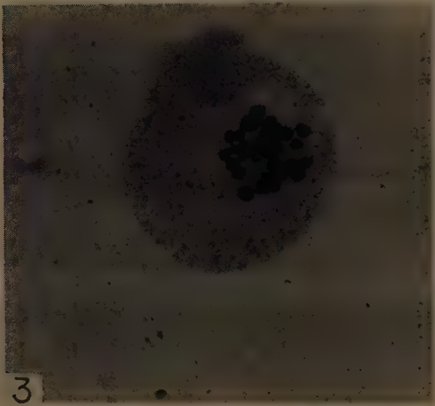
About 20 to 30 ml. of the collected fluid was centrifuged, and the deposit was fixed and stained with aceto-orcein. Most of the effusions, as well as the malignant cells, also contained benign types: macrophages, polymorphonuclear leucocytes, and mesothelial cells in varying proportions. Usually, there were a large number of cells, including tumor cells, in various stages of degeneration. Comparison of the cell-composition of fluids has shown a continuous shift in the proportion of the various cell types and in the number of degenerating cells. A similar phenomenon was seen in experimental ascites by Goldie and Felix (1951). The changes are very likely the result of alterations in the physiological or nutritional conditions of the ascitic fluid.

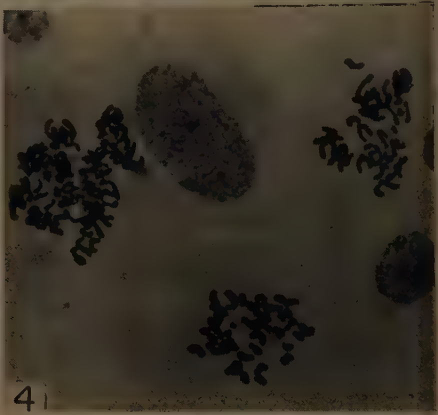
Only 26 of the 98 effusions were suitable for detailed study. This was because many of the effusions had very few or no dividing cells, and several effusions with very high number of dividing cells had to be discarded owing to bad fixation of the chromosomes.

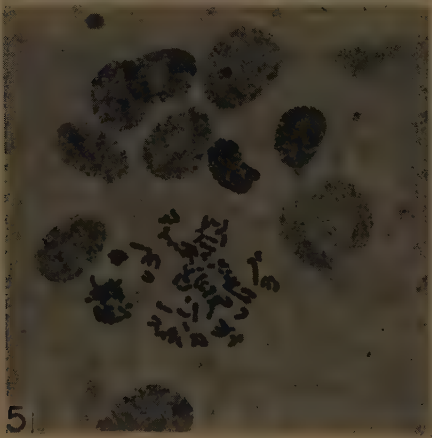
Our investigation has shown that the malignant cell population of human ascitic fluids contains both isolated cells and cell aggregates (FIGURES 1 and 2). These aggregates are of two types. One is composed of cells with well-defined cell membranes (henceforth to be referred to as "free-cell" ascites). The other contains colonies of cells in which the cell boundaries are indistinct or absent.











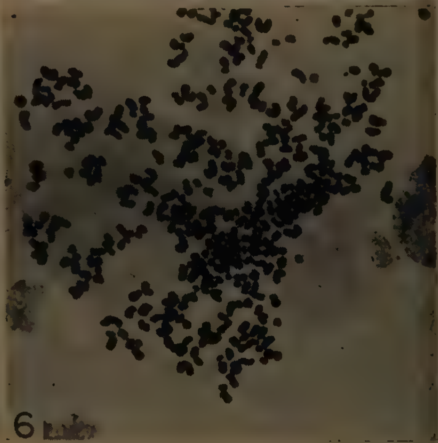


FIGURE 1. Microphotograph showing a group of malignant cells in "free-cell" ascites. The individuality of cells is preserved. ×2000.

FIGURE 2. Microphotograph illustrating a section of a cell colony in which cell boundaries are indistinct. ×2000.

FIGURE 3. "Ball-shaped" chromosomes in an isolated malignant cell of free-cell ascites. ×2000.

FIGURE 4. Synchronous division of cells in a colony-forming ascites. The three cells in division show differences in chromosome contraction. ×2000.

FIGURE 5. Mitosis in a hyperdiploid cell with 32 chromosomes. The structure of chromosomes is distinct. ×2000.

FIGURE 6. Mitosis in a highly polyploid cell with hexaploid (288 ?) chromosome number. The chromosomes are greatly contracted. (FIGURES 5 and 6 illustrate divisions in cell colony-forming ascites.) ×2000.

Isolated cells occur with both types of aggregates, but they represent only a small proportion of the total of malignant cells.

Chromosome Variability in Free-Cell Ascites

Cell individuality is always maintained in the aggregates of "free-cell" ascites. The number of cells that can associate varies between 2 and 80. The diameter of tumor cells varies from 20 to 50 μ. It was found that the members of the cell groups may differ in size, while the diameter of their nuclei is nearly the same. The different size very likely indicates differences in metabolic activity.

The frequency of dividing cells in the free-cell effusions is rather low. On the average, it is 8 per 1000 cells. Division is rare in single cells, but more common in the cells of an aggregate. The chromosomes of single cells usually exhibit supercontraction, and they resemble the "ball-shaped" chromosomes of human spermatogonial cells (Koller, 1936) and of certain experimental ascites (Tjio and Levan, 1954). See FIGURE 3.

The number of chromosomes was counted in free-cell ascites of three patients, and the data are shown in TABLE 1. Although the total number of cells analyzed in each case is small owing to technical difficulties, it can be seen that by far the largest group has the diploid chromosome number of 48. There are a few cells with subdiploid numbers, and few are polyploid.

The range and frequency of chromosome variation in the three cases was compared, using a statistical method. The estimation of similarity or dissimilarity was based on the assumption that the coefficient of variation for a series of nonnormal curves will itself tend toward a normal distribution. It was found that the differences in dispersion between the three effusions do not approach statistical significance (P is approximately 0.5).

Chromosome Variability in Ascites with Cell Colonies

The most characteristic feature of these ascites is the absence of cell boundaries in the colonies. The nuclei appear to lie within a common cytoplasm. The number of nuclei may be as high as 200, but the average number is around 80. Mitosis is frequent in the cell colonies (FIGURE 4), which usually contain a varying number of degenerated nuclei. The diameter of nuclei varies from 7 to 35 μ, the range of variation being related to the size of colony. The greater the number of nuclei within a colony, the greater is the variation in nuclear size. There is also a relationship between the frequency of mitosis within the colony and its size. While the rate of mitosis is 10 to 15 per cent in colonies in which the number of nuclei is not more than 30, the rate drops rapidly to 1 per cent or less in those that contain more than this number.

Five effusions with cell colonies have been studied cytologically, and the data obtained in three cases are presented in TABLE 2. The three cases illustrate the kind of variation in chromosome numbers that one may find in this type of ascites. In the first case (No. 021953), the peritoneal effusion was associated with carcinoma of the ovary, and a very large number of excellently preserved mitosis was available for analysis. The counting was facilitated by

TABLE 1

CHROMOSOME NUMBERS IN FREE CELL ASCITES

Case (age and sex)	Carcinoma	Location	Interval between paracenteses in days	Quantity of effusion in liters	Chromosome number									Total No. of cells analyzed
						36	40	42	43	48	50	>80	>100	
F. B. 33 yrs. ♀ 021684 (see FIGURE 7)	Ovary	Peritoneal cavity	32	3	No. %	1 4.0	— —	2 8.0	— —	17 68.0	1 (?)* 4.0	4 16.0	—	25 (5)*
M. M. 70 yrs. ♀ 028805	Primary is not known	Pleural cavity	11	1.5	No. %	— —	2 6.7	1 3.3	— —	24 80.0	— —	1 3.3	2 6.7	30 (3)
L. P. 55 yrs. ♀ 644-38	Breast	Peritoneal cavity	40	4.5	No. %	— —	— —	— —	— —	27 90.0	— —	2 6.7	1 3.3	30 (3)

* Numbers indicated with (?) and numbers in ( ) indicate the number of cells in which the count of chromosomes is uncertain.

the fact that the metaphase chromosomes in hyperdiploid and polyploid cells were very short, resembling chromosomes observed after colchicine treatment (FIGURES 5 and 6). Only 7 of the 64 mitoses (or 10.9 per cent) were diploid, 36 (56.1 per cent) hypodiploid, 21 (32.7 per cent) hyperdiploid, and 14 (or 21.8 per cent) high polyploid. In this effusion, there is thus a wide variation in chromosome number, as has been observed in certain experimental ascites by Levan and Hauschka (1953).

In the histogram of FIGURE 7, the variation in this case (No 021953) is compared with that in one of the free-cell ascites already mentioned (No. 021684, TABLE 1). The primary carcinoma of the free-cell ascites was anaplastic and that of the ascites with cell colonies was reported as an adenocarcinoma. The ascitic fluid of case No. 4-43, which was aspirated from the pleural cavity, is of special interest because of the very high frequency of polyploid and multinucleate cells. About one third of the mitoses fall into this category. Statistical analysis has shown that the case is similar to that described previously in respect of the dispersion of chromosome classes (P = 0.2).

The third effusion was aspirated from the pleural cavity of a patient with cancer of the lung. It had an unusually high number of diploid cells (58 per cent) while the frequency of polyploid cells was low (9.7 per cent). The distribution of chromosome numbers was different from that observed in the two previous effusions, and this difference was statistically significant (P = 0.0001 and 0.001 respectively). Furthermore, the size of cell "colonies" was found to be rather uniform, the average number of nuclei in a cell group being about 20 to 25. Mitosis was infrequent in the colonies, and few abnormal divisions were seen. Closer analysis by phase-contrast microscopy disclosed cell boundaries in the aggregates. The findings suggested that the third effusion repre-

TABLE 2
CHROMOSOME NUMBER IN ASCITES-FORMING CELL COLONIES

Case (age & sex)	Carcinoma	Location of effusion	Interval between paracenteses in days	Quantity of effusions in liters		Chromosome numbers																							Total No. of cells analyzed
						25	28	30	32	33	34	36	37	38	40	41	42	44	46	47	48	50	53	56	62	63	>80	>140	
A. V. 45 yrs. ♀ 021953 (see FIGURE 7)	Ovary	Peritoneal cavity	48	9	No.	1	5	1	4	2	4	3	2	4	2	3	—	2	2	1	7	2	1	2(?)*	1	1(?)	5	9	64
					%	1.6	7.8	1.6	6.3	3.1	6.3	4.7	3.1	6.3	3.1	4.7	—	3.1	3.1	1.6	10.9	3.1	1.6	3.1	1.6	1.6	7.8	14.1	(17)*
M.A.L. 74 yrs. ♀ 4-43	Breast	Pleural cavity	7	1	No.	—	—	—	—	—	—	—	1	—	—	—	2	1	2	—	10	1	2	1	1	2(?)	5	4	32
					%	—	—	—	—	—	—	—	3.1	—	—	—	6.3	3.1	6.3		31.2	3.1	6.3	3.1	3.1	6.3	18.6	12.5	(11)
C.M. 57 yrs. ♂ 021226	Bronchus	Pleural cavity	7	0.8	No.	—	—	—	—	—	3	1	1	—	—	—	—	—	1	1	18	1	2	—	—	—	3		31
					%	—	—	—	—	—	9.7	3.2	3.2	—	—	—	—	—	3.2	3.2	58.0	3.2	6.5	—	—	—	9.7		(5)

* See TABLE 1.

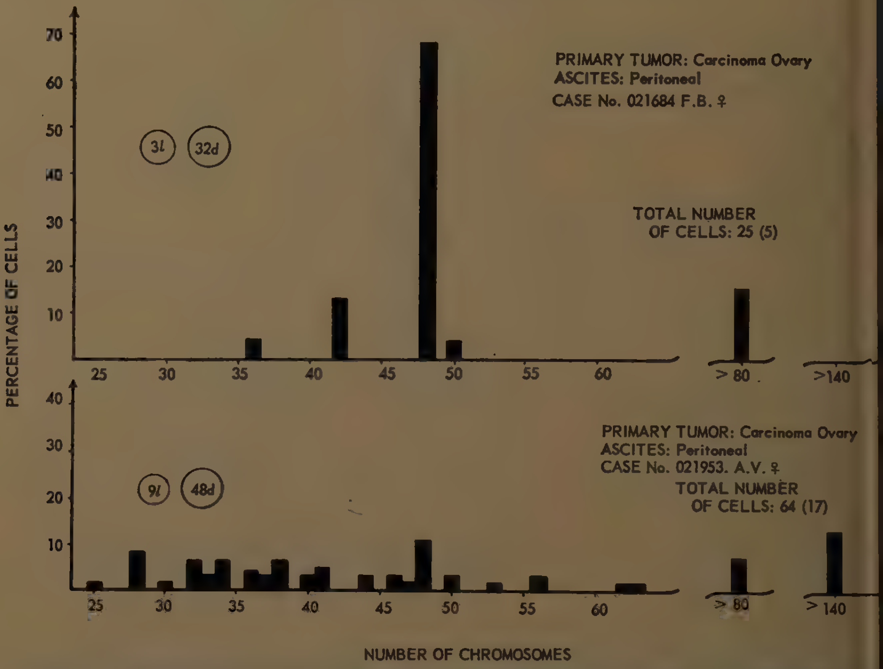


FIGURE 7. Histograms showing the distribution of chromosome numbers in a "free-cell" ascites (case No 021684) and "cell colony"-forming effusion (case No. 021953). Numbers in circles show the quantity of flui (in litres) and the time interval in days between the successive paracenteses. Numbers in brackets indicate th number of cells in which counting of chromosomes was uncertain.

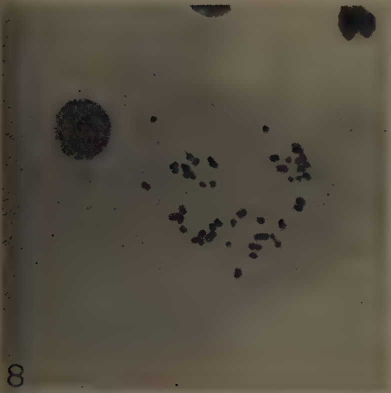
sents a transitional type between the free-cell ascites and those fluids in which cell colonies are formed.

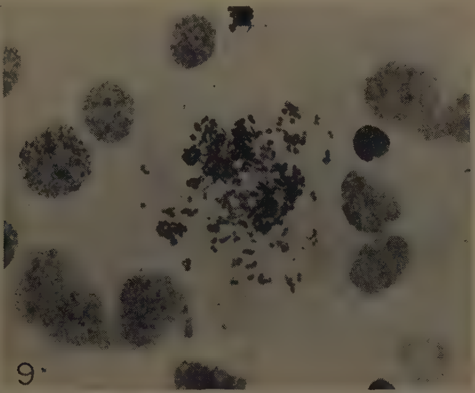
Variation in Chromosome Morphology and Behavior

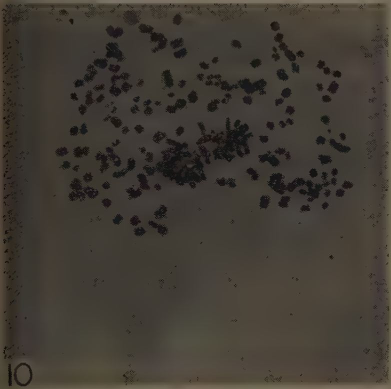
In only two out of the 98 ascitic fluids in the present investigation were the cells highly uniform as regards their chromosome numbers and chromosome morphology, and normal in chromosome behavior. In some of these cells, it was possible to locate the position of the centromere in most of the 48 chromosomes, and the observations made agreed with those of Hsu (1954), who studied the structure of human chromosomes in tissue culture.

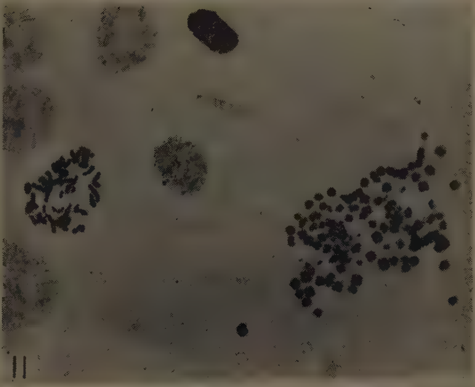
In all the rest of the human effusions studied, particularly in the cell-colony forming type, there is a large number of cells exhibiting the most diverse abnormalities, some of which will be described below.

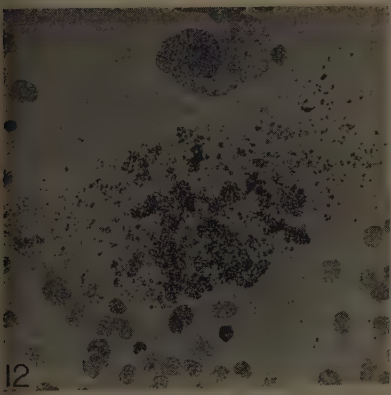
(1) *Contraction.* Excessive shortening of the chromosomes is one of the most common abnormalities seen in human effusions. The chromosomes appear as short, thick, rodlike bodies in which the primary constrictions cannot be recognized (FIGURES 8, 10, 11). Adjacent cells were found that differed in the degree of chromosome contraction. The abnormal shortening of chromosomes is of great advantage technically, as it makes the counting in hyperdiploid or high polyploid cells an easier task than it would be otherwise (FIGURES 10 and 11).











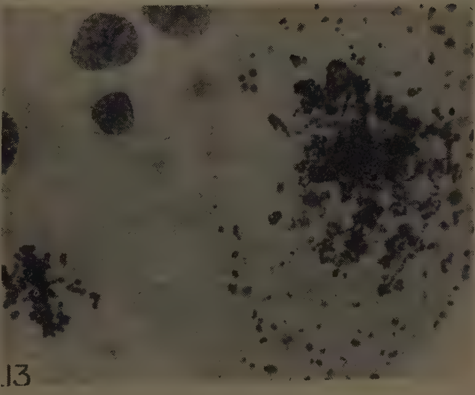


FIGURE 8. Mitosis in a hypodiploid cell (44 chromosomes) showing excessive contraction of chromosomes. ×2000

FIGURE 9. Chromosome bridges and acentric fragments at anaphase in cell colony-forming ascites. ×2000.

FIGURE 10. Division of a hypodiploid and a polyploid cell in close proximity to each other. Chromosomes of the former are in side view. The chromosomes of the polyploid cell (140 ?) are greatly contracted. ×2000.

FIGURE 11. Two dividing cells in a cell colony-forming ascites, showing differences in chromosome number and in chromosome contraction. ×2000.

FIGURE 12. Chromosome disintegration in a giant, highly polyploid cell. ×180.

FIGURE 13. Superfragmentation of chromosomes in a polyploid cell. The acentric fragments are scattered in the cytoplasm, while the centric chromosome segments show incomplete congression on the equatorial plate (microphotograph shows only part of the giant cell). ×2000.

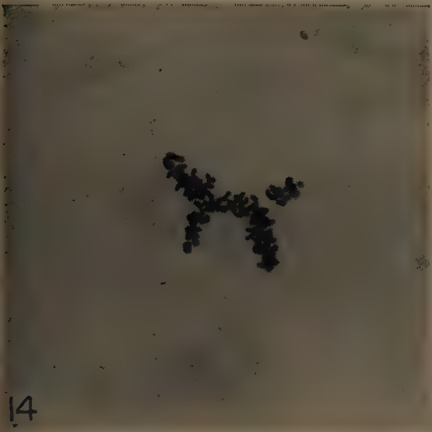
(2) *Structural changes.* Chromosome bridges and acentric fragments at anatelophase were encountered in some cells. They are assumed to be due to structural changes in the chromosomes (FIGURE 9). Several interphase cells were found with micronuclei, suggesting that chromosome breakage or fragmentation occurs in the cells of ascitic fluids. It is interesting to note, however, that this type of abnormality is rather rare, and was completely absent from many of the effusions in the present study.

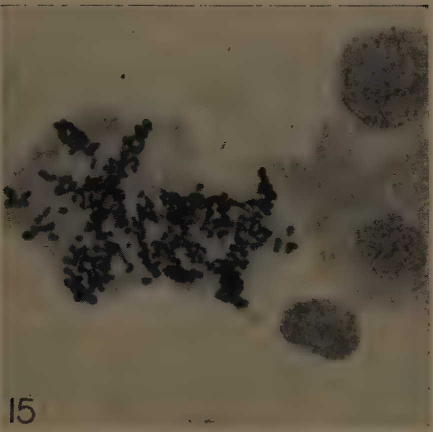
(3) *Chromosome disintegration.* This phenomenon can be described as superfragmentation of the chromosome. It usually occurs in highly polyploid cells (FIGURES 12 and 13). The larger centric segments of chromosomes may form a metaphase group and enter into resting stage, while the small acentric fragments are scattered throughout the cell. More often, however, the disintegration is complete and the cell degenerates (FIGURE 12). A very similar abnormality was observed by Hsu (1954) in the tissue culture of human tumor. He found cells in mitosis "with great numbers of fragmentary particles." In some instances, the failure of reconstruction of interphase nucleus at the end of mitosis has been observed. The phenomenon is very similar to that described by Levan and Hauschka (1953) in a lymphosarcoma-ascites of mice.

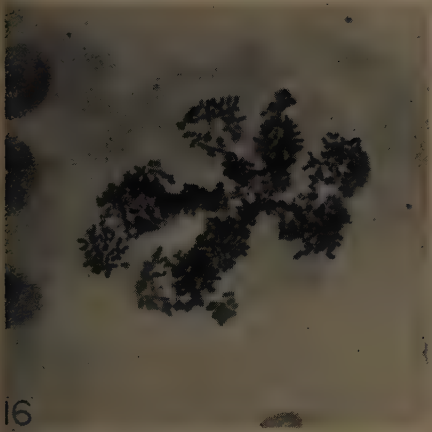
(4) *Spindle abnormalities.* These are the most common anomalies in cells of experimental ascites (*e.g.*, tetraploid Ehrlich and Yoshida sarcoma). They were also frequently observed in human malignant effusions (FIGURES 14 to 17). Spindles with 3, 4, and more poles (or centrosomes) were found in both free-cell ascites and in effusions with colonies. It seems that spindle aberrations are more common in the latter than in the former type of effusion. There is some evidence to indicate that the spindle mechanism is incomplete or absent, which may be partly responsible for the supercontraction of metaphase chromosomes and for the formation of polyploid nuclei (FIGURE 17).

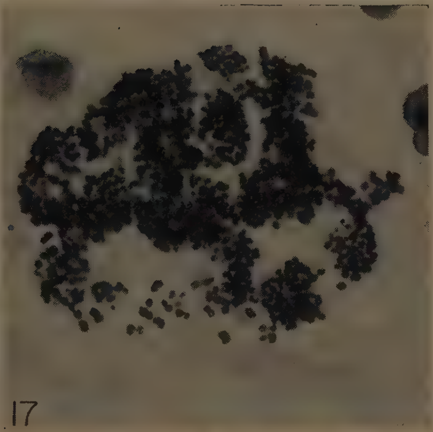
(5) *Giant cells.* These cells can be grouped according to the number of nuclei present. There are "uninucleate" cells with large nuclei, which are probably highly polyploid. In a few dividing giant cells, more than 400 chromosomes were counted, although this counting was incomplete. Giant cells are usually present in groups of 2 to 8 (FIGURE 12), which suggests that they have been derived from one common parental cell. The shape of nuclei of some giant cells is irregular, with numerous lobes, which strongly suggests that they might have arisen by the fusion of several sets of chromosomes, each from a single nucleus. There was no evidence from the present work that endomitosis or endoreduplication is the process by which giant, polyploid cells came about in those human effusions. A similar observation was reported by Tanaka (1953) on regenerating liver of the rat.

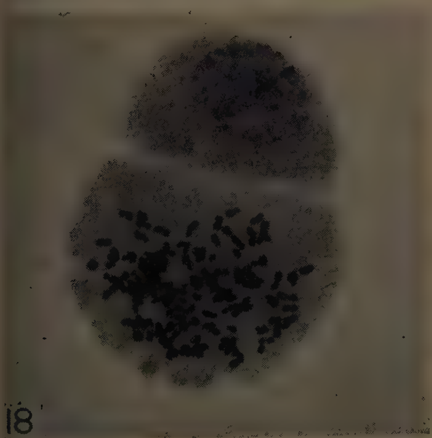
"Binucleate" cells are very common in free-cell ascites. They may show an incomplete membrane between the two potential cells (FIGURE 18). In most of the cases, however, the membrane is absent and the cell can be described as a true binucleate. Division of the two nuclei is not necessarily a synchronous process. The size of the two nuclei may differ, and lack of synchronization may be due to differences in chromosome numbers. Instances were found in which the chromosomes of one nucleus disintegrated during mitosis (FIGURE 19).











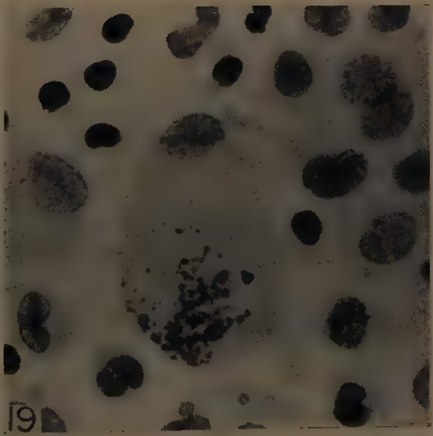


FIGURE 14. Mitosis with quadripolar spindle, which results in the formation of aneuploid cells. ×2000.

FIGURES 15, 16, 17. Mitosis in polyploid and multinucleate cells showing the formation of multiple-equatorial plates. ×2000.

FIGURE 18. Division in a doublet or incomplete binucleate cell; the dividing partner is polyploid (>120). ×2000.

FIGURE 19. Mitosis in a true binucleate cell, in which the chromosomes undergo degeneration. ×2000.

"Multinucleate" cells have between 4 and 18 nuclei, which may be of the same size or may differ particularly in cells in which their number is high. The nuclei are sometimes connected by thin chromatin bridges, and cells with such nuclei closely resemble the megakaryocytes of bone marrow in rodents.

The cytological abnormalities described above are the most common types found in malignant cells of human effusions. In three instances, it was possible to compare cell behavior between the primary tumors and effusions. The incidence of dividing cells with abnormalities was much higher in the latter than in the primary tumor, from which the cells of the effusion had originated. Furthermore, while the most common anomaly in primary tumor was chromosome stickiness, it was very infrequent in effusions. It seems that the different environment at the two sites may be the cause of differences observed in cell behavior.

The nutritional conditions in the ascitic fluid undergo continuous, and probably rapid, changes and, as the volume of fluid increases, the proportion of various cell types alters, indicating that the environment becomes less favorable for certain types. It was furthermore found that the quantity of fluid and the interval between successive paracenteses are important factors that influence the frequency of cells in mitosis and that of the mitotic abnormalities. Some observations have also been made by the author on experimental ascites of mice and rats, and evidence was obtained that suggests that cells in a 12-day-old ascitic fluid had a higher incidence of abnormality than cells in a 6-day-old effusion. The importance of environment in affecting cell behavior was particularly demonstrated in the case of one carcinoma of the ovary that was associated with peritoneal and pleural effusions. In the latter, only 5 per cent of dividing cells were abnormal while, within the peritoneal cavity, the incidence was 20 per cent.

Chromosome Variation in Successive Effusions of the Same Patient

When comparing cell population of successive effusions, it must be borne in mind that fluid from the body cavities can never be completely removed by aspiration. A variable amount of this "old" fluid with its cell population is always left, and it contributes to the new pool and may also influence the frequency distribution of the various cell types. It was found, furthermore, that metastatic infiltrations and adhesions on the serous membrane are important sites from which cells and cell aggregates can be cast off into the effusion. In any comparison between different ascites, it must be borne in mind that the quantity of fluid and the length of the interval between successive paracenteses cannot be standardized. Our investigation has shown that these have an influence on the development of cell variation.

In the present investigation, seven effusions very suitable for cytological analysis were aspirated from three patients. Histograms of FIGURE 20 show the cell polymorphism in three successive ascites of one patient with carcinoma of the cervix. The cells of these effusions exhibited a rather wide range of variation in chromosome number. Though the dispersion of the various cell-classes was similar ($P = 0.4$), in the second effusion, about 25 per cent of the

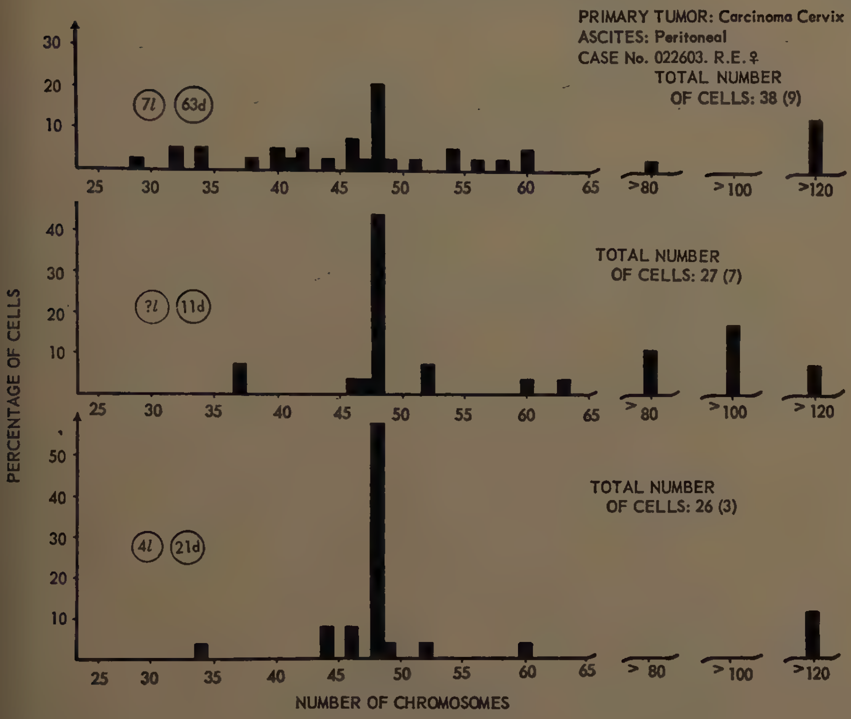


Figure 20. Distribution of chromosome numbers in three successive peritoneal effusions of a patient with carcinoma of the cervix.

total cell population analyzed was highly polyploid, while the third effusion showed a high frequency of diploid cells.

Data from two other patients are given in TABLE 3. Case No. 023647 was a carcinoma of the breast with a cell-colony forming ascites, and case No. 021356 was a carcinoma of the uterus, associated with a free-cell effusion. The spectra of chromosome variability found in the two effusions of the patient with breast carcinoma do not differ ($P = >0.5$), while the two effusions of case No. 021356 show a statistically significant difference ($P = <0.001$) in the distribution of chromosome numbers. This finding is very remarkable in view of the fact that there was an interval of only four days between the two paracenteses. It is necessary to mention that the second effusion of this patient contained few dividing cells, and that almost half of them were unsuitable for analysis owing to bad fixations. Thus the difference found in the last case may not be a real one.

A comparison of cell behavior of successive effusions brought forward further evidence of the importance of the interval between their aspiration on cell composition. The data strongly suggest that the polyploid class increases with the "age" of the fluid. Confirmatory evidence that this may be of common occurrence on experimental ascites has been obtained by the author in effusions of the hyperdiploid and tetraploid Ehrlich and Yoshida sarcoma.

TABLE 3
CHROMOSOME NUMBER ON SUCCESSIVE EFFUSIONS FROM THE SAME PATIENT

Case (age & sex)	Carcinoma	Location of effusion	Interval between paracenteses in days	Quantity of effusion in liters	Chromosome number																					Total number of cells analyzed
						32	33	34	35	36	38	40	42	46	47	48	49	50	52	54	58	63	<80	<100	<120	
M.B.	Breast	Peritoneal cavity	14	3	No.	1			1				3		1	*6*		2		2	1		3	2	3	25
55 yrs.					%	4.0			4.0				12.0		4.0	*24.0*		8.0		8.0	4.0		12.0	8.0	12.0	(8)
♀			28	7.5	No.		1	2		2		3		1		*12*			1(?)	2			4	6	5	39
023647					%		2.6	5.1		5.1		7.7		2.6		*30.8*			2.6	5.1			10.2	15.4	12.8	(16)
E.C.	Uterus	Pleural cavity	30	1.5	No.	1								2		*13*				1		1(?)	2	3	3	26
64 yrs.					%	3.8								7.7		*50.0*				3.8		3.8	7.7	11.5	11.5	(9)
♀			4	0.5	No.						2					*18*	1						1			22
021356					%						9.1					*81.8*	4.5						4.5			(2)

* See TABLE 1.

Cytological Changes in Ascitic Fluids After Au 198 Treatment

During the present investigations, 32 effusions treated with radioactive colloid gold were available for analysis. Au 198 emits β radiation of low penetrating power, 75 per cent of which is delivered within five days (half life of Au 198 is 2.7 days). The dose usually administered was 120 mc (about 2500 rep). The colloid particles of radioactive gold are preferentially taken up by macrophages, and much of it is deposited on the serous membranes. A certain amount, however, has been identified in the cytoplasm of tumor cells. In this respect, malignant cells of human effusions differ from those of experimental ascites of sarcoma 37 in mice. According to Goldie and his co-workers (1950) only the macrophages contained radioactive gold particles in this mouse ascites.

Without going into a detailed description of the general effect of Au 198 in human carcinomatosis, which will be published elsewhere, some of the cytological changes seen in effusions under treatment may be mentioned here. The earliest sample was aspirated from a patient with carcinoma of the ovary six days after the administration of Au 198. Many tumor cells had grossly enlarged nuclei, some of which contained colloid gold particles. Dividing cells were absent, and about 8 per cent of cells in interphase had 2 to 6 micronuclei of varying size.

The best observation, however, was made on an ascites 21 days after treatment. It contained a large number of dividing cells, and 23 of the 142 mitoses analyzed were found to contain many acentric fragments indicating radiation effect on the chromosomes. Most of the dividing cells were free of colloid gold particles. This indicates that injury to the chromosomes was caused by extracellular β-radiation. Experience gained by analyzing radiation-induced chromosome fragmentation in various human carcinomas enabled the author to estimate the probable dose of radiation received by the tumor cells in effusion. By taking into consideration the number of injured cells, the amount of chromosome fragmentation per cell, and the interval between administering Au 198 and fixing the cells for analysis, the conclusion was drawn that cell damage was caused by a dose of 1000 to 1200 r. In view of the fact, however, that, in certain experimental ascites, the free tumor cells were found to be at least five times more sensitive than the cells of the solid tumor (Lasnitzky 1954), it is possible that the actual dose received was less than estimated.

In a number of instances, the cell composition of effusions was compared before and after treatment. It was found that in some cases the malignant cell population had been almost completely destroyed by the treatment while, in others, the proportion of tumor cells had been greatly reduced, with or without change in cell polymorphism. TABLE 4 illustrates the cytological variation observed in three effusions of a patient treated twice with Au 198. In this particular case, the cell polymorphism of the third effusion was significantly different from that shown by the cells of the two effusions obtained previously ($P = 0.004$ and 0.001 respectively). The histograms of FIGURE 21 clearly demonstrate the shift in cell composition.

The most significant change in effusions seen after radioactive colloid gold treatment is the increase of the frequency of diploid cells. If cells with the

TABLE 4

CHROMOSOME NUMBER IN EFFUSIONS BEFORE AND AFTER AU 198 TREATMENT

Case (age & sex)	Carcinoma	Location of effusion	Interval between paracenteses in days	Quantity of effusion in liters	Treatment		Chromosome number																							Total No. of cells analyzed
							32	34	35	36	38	40	42	43	44	45	47	48	49	50	52	53	54	55	56	63	>80	>100	>120	
E.P. 56 yrs. ♀ 024137 (see FIGURE 21)	Ovary	Peritoneal cavity .	16	8.5	Nil	No.	1			1		1	3			1		*14*	1		2	1	1		2	2(?)*	2	1	3	38
						%	2.6			5.3		2.6	7.9	2.6		2.6		*36.5*	2.6		5.3	2.6	2.6		5.3	5.3	5.3	2.6	7.9	(8)*
			20	2.5	120 mc. Au 198	No.		2	1(?)		1		2				2	*18*		1			1				2	1	3	34
						%		5.9	2.9		2.9		5.9				5.9	*52.9*		2.9			2.9				5.9	2.9	8.8	(7)
			27	4.5	120 mc.	No.									1			*22*	1						2	1(?)		2		29
					Au 198	%									3.4			*75.9*	3.4						8.9	3.4		6.9		(3)

* See TABLE 1.

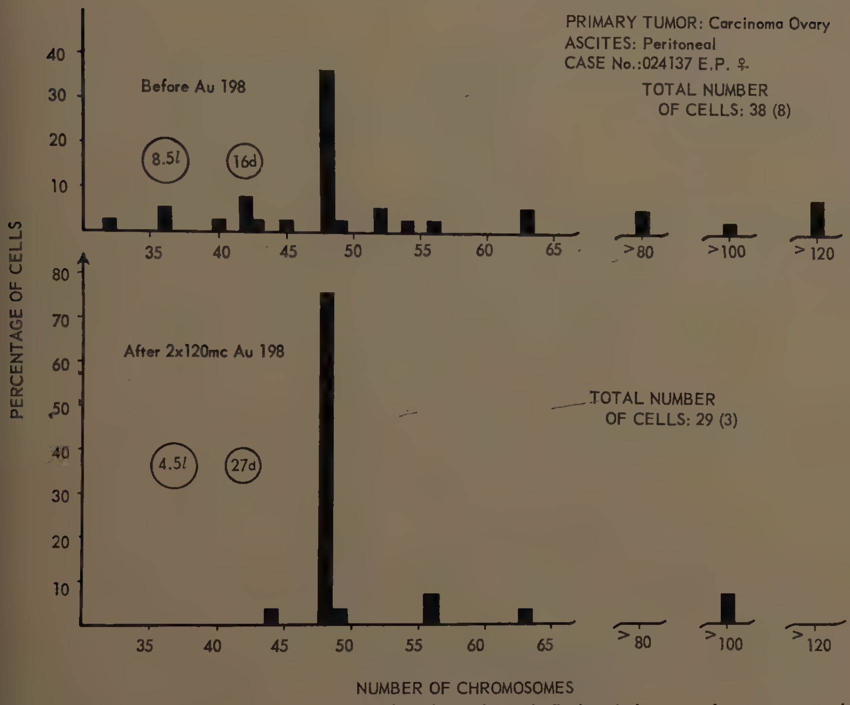


Figure 21. Distribution of chromosome numbers in peritoneal effusions before and after treatment with Au 198.

diploid chromosome number represent the "stem cells" of ascites, *i.e.*, the cells that are the main source for the cell population of the new effusion, then our data seem to indicate that the "stem cells" are more resistant to Au 198 than cells with hypodiploid or hyperdiploid chromosome number. This was found by Makino and Tanaka (1953) to be the case in certain mouse ascites treated with podophyllin. It is probable, however, that the higher frequency of diploid cells in effusions after treatment with Au 198 may be due to the fact that a variable proportion of the new cell population is supplied from local metastatic adhesions, which were found to be composed mainly of diploid cells.

Comparison of Cell Polymorphism in the Peritoneal and Pleural Fluids

During the investigation, it was observed that the cell population of pleural fluid is more heterogeneous or mixed than that of the abdominal effusions. In the former, the various types of inflammatory cells and mesothelial cells of the serous membranes are all represented, and the proportion of each type is high (20 to 30 per cent). In the peritoneal fluid, on the other hand, usually only one or two benign cell types beside the tumor cells appear in an appreciable proportion, and the frequency of all other types is very small (less than 5 per cent). Furthermore, it was noticed that malignant cells in the pleural fluid exhibit a greater degree of independence, and the effusions are usually of the

TABLE 5

CHROMOSOME NUMBER IN PERITONEAL AND PLEURAL EFFUSION FROM THE SAME PATIENT

Case (age & sex)	Carcinoma	Location of effusion	Interval between para-centeses in days	Quan-tity of effusion in liters		Chromosome number																		Total number of cells ana-lyzed
						32	34	35	38	40	41	43	44	45	46	48	50	51	52	56	59	>80	>100	
E.C. 66 yrs. ♀ 002741 (see FIGURE 22)	Ovary	Peritoneal cavity	12	4.5	No.	3	1	1	2	1	2		2	1		*23*	5	2	2	2	1(?)*	3	2	52
					%	5.9	1.9	1.9	3.8	1.9	3.8		3.8	1.9		*44.4*	9.4	1.9	3.8	3.8	1.9	5.9	3.8	(6)*
	Naso-pharynx	Pleural cavity	32	0.85	No.							1	2	1	1	*24.0*	1	2		1(?)		1		34
					%							2.9	5.9	2.9	2.9	*70.6*	2.9	5.9		2.9		2.9		(2)

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* See TABLE 1.

"free cell" type. In the peritoneal cavity, the cell colony-forming ascites are the most common type.

It is known that, in the two serous cavities, beside the anatomical peculiarities, the physiological conditions are different. It may be assumed therefore, that the different behavior is due to the locality; *i.e.*, cell behavior is environmentally conditioned. Interesting observations relevant to this aspect have been reported by Goldie *et al.* (1952). For this reason, it seemed to be particularly important to analyze cell populations of pleural and peritoneal effusions in the same patient. During the present investigation, 12 patients were found having double ascites, one in the pleural and another in the peritoneal cavity. The data obtained by studying double effusions are meager and not sufficient for a quantitative comparison of the respective cell populations, yet certain facts emerged that are of special interest.

Generally, it was found that, apart from the variable proportion of benign cells, the type of the effusion and the polymorphism of the malignant cells were similar in both localities with one exception: case No. 002741. The data from the cytological analysis of this particular case are given in TABLE 5 and the chromosome polymorphism is illustrated by the histograms of FIGURE 21. Although no satisfactory statistical analysis of the data could be made, owing to the small number of cells counted and the large and indefinite deviation of the cells with a high chromosome number, the malignant cell populations of the double effusions exhibited different features. The peritoneal ascites was the cell colony-forming type with 6 per cent of the dividing cells having spindle abnormalities. The incidence of polyploid cells was nearly 10 per cent. In the pleural cavity, the ascitic fluid was clearly of the "free cell" type in which the diploid cells were the most numerous. Mitotic disorder was absent, and the incidence of polyploidy low (3 per cent). Furthermore, the cells of the two effusions showed differences in the nuclear structure. In the pleural ascites, the cell nuclei had numerous small Feulgen-positive chromocenters, while those in the peritoneal fluid had only one large center, usually associated with the nucleolus. Post-mortem investigation revealed the presence of *two primary carcinomata* in this patient, namely adenocarcinoma of the ovary and an anaplastic lymphosarcoma of the nasopharynx.

Thus, our studies on 12 patients with double effusions demonstrated that (1) the type of ascites and cell behavior cannot be attributed to environmental conditions only; (2) in cases where tumor cells of the effusions show differences in behavior and morphology, the presence of two primary tumor sites should be assumed.

Cell Behavior in Primary Tumor and Ascites

Biopsy specimens from tumors at the primary sites on which diagnosis has been made were available in 15 out of the 56 patients with effusions. The histopathological type of the primaries and the cytological type of effusion are shown in TABLE 6. It seems that differentiated carcinomas are associated with cell colony-forming ascites, while the cells of dedifferentiated or anaplastic primary tumors usually appear in the effusions as free cells or independent

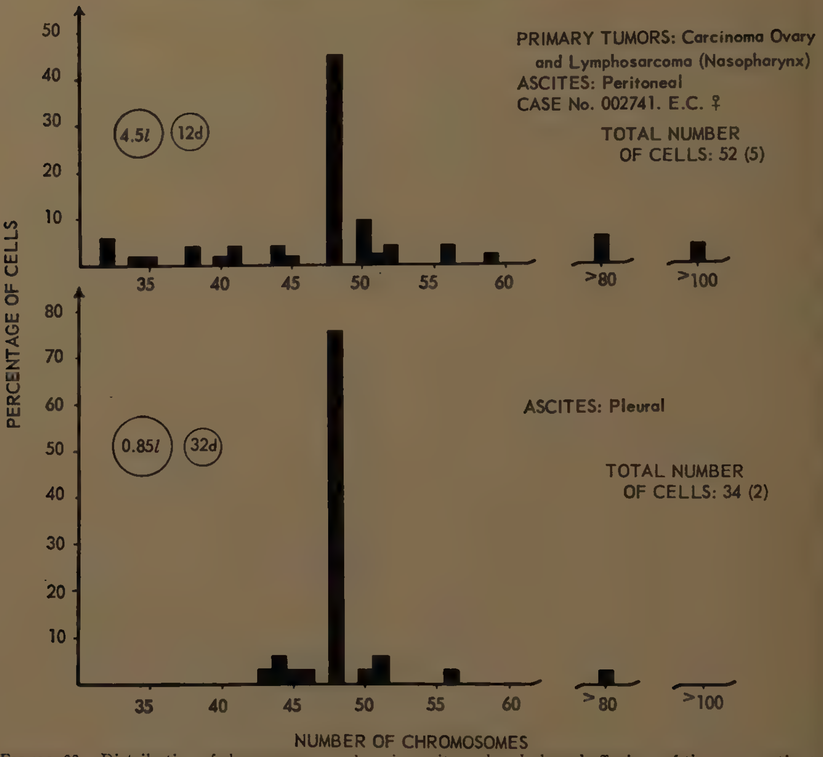

FIGURE 22. Distribution of chromosome numbers in peritoneal and pleural effusions of the same patient.

components of cell aggregates. This relationship is particularly clear in the case of ovarian adenocarcinomas with effusion in the peritoneal cavity. It is less clear in the case of carcinoma of the breast and lung with pleural effusion, owing to the large variety of benign cells present.

In one case, the primary tumor was reported to be a well-differentiated adenocarcinoma of the ovary. The initial diagnosis was based on a biopsy specimen obtained 18 months before the appearance of the peritoneal effusion. The cell population of the ascites was found to be of the "free cell" type, most commonly observed in the presence of anaplastic primary tumors. Post-mortem examination four months later showed that both the "primary" tumor and metastatic infiltrations on the serous membranes were of the *anaplastic* type. This observation is of special significance since it shows that (1) dedifferentiation at the primary site can occur, and that (2) cell behavior in the effusion is closely related to the primary tumor.

Chromosome counts in the "solid" primary tumors referred to in TABLE 6 were not possible, and we have only indirect evidence provided by the measurement of nuclear volume that most of the cells of the tumor tissue were diploid. During the past two decades, the author has studied cytological behavior in a great variety of carcinomata of the skin, cervix, ovary, lung, *etc.* and, while

TABLE 6

PRIMARY TUMORS AND CELL BEHAVIOR IN EFFUSIONS

Case (sex & age)	Primary tumor		Effusion		Notes
	Site†	Type	Site†	Type	
021194 ♀ 52 yrs.	O.	Anaplastic carc.	P.	free cells	Transitional cell type.
021684 ♀ 33 yrs.	O.	Anaplastic carc.	P.	free cells	PM. *cf.*, TABLE 1
021741 ♀ 45 yrs.	O.	(Cystic-mucoid) adenocarcinoma	P.	cell colonies	
021911* ♀ 59 yrs.	O.	Papillary adenocarcinoma	P.	free cells	PM.-anaplastic
023984 ♀ 63 yrs.	O.	Papillary cystadenoma	P.	cell colonies	
024137 ♀ 56 yrs.	O.	Cystadenoma	P.	cell colonies	*cf.* TABLE 4
002741 ♀ 66 yrs.	O.	Adenocarcinoma ?	P. + Pl.	cell colonies free cells	PM. *cf.*, TABLE 5 lymphosarcoma nasopharynx.
022603 ♀ 53 yrs.	C.	Diff. squamous cell	P. + Pl.	cell colonies cell colonies	
021356 ♀ 64 yrs.	U.	Adenocarcinoma	Pl.	cell colonies	*cf.* TABLE 3.
021226 ♂ 57 yrs.	Bro.	Anaplastic (spindle cell)	Pl.	Mixed (free cells)	PM. *cf.*, TABLE 2
023671 ♀ 50 yrs.	Bro.	Anaplastic carc.	Pl.	free cells	
644-38 ♀ 55 yrs.	Bre.	Diffuse, pleomorphic anaplastic	P. Pl.	free cells mixed (free cells)	
4-43 ♀ 74 yrs.	Bre.	Adenocarcinoma	Pl.	cell colonies	PM. *cf.*, TABLE 2
1481-39 ♀ 63 yrs.	Bre.	Scirrhous-spheroidal anaplastic carc.	Pl.	free cells	
023727 ♀ 59 yrs.	Bre.	Acinar-adenocarcinoma	P.	cell colonies	

* At post-mortem, anaplastic carcinoma was found at the primary site.

† O. = ovary, C. = cervix, U. = uterus, Bro. = bronchus, Bre. = breast, P. = peritoneal, Pl. = pleural.

tumors were encountered in which polyploid and multinucleate giant cells were numerous, aneuploidy (*i.e.*, deviations from the diploid number involving few chromosomes only) was rare. By using microspectrophotometry on five mouse tumors, Bader (1953) arrived at the conclusion that tumor tissue is largely composed of diploid cells or their multiples. In tumors, the scatter of DNA content from cell to cell is greater than that seen in normal tissues and is attributed to the higher frequency of mitoses and not to aneuploidy (Leuchtenberger *et al.*, 1954). The frequency of hypodiploid or hyperdiploid cells reported by Hsu and Pommerat (1953) and Beatty (1954) in normal and malignant tissues is usually below the incidence observed by the author in the present investigation.

In only one case has chromosome polymorphism been found in a primary tumor that closely resembles that seen in human effusions (Koller, 1947). An adenocarcinoma of the large intestine involving the colon and the mesentery had a mucinous region composed of hypodiploid cells only, with 32 to 34 as the mean number of chromosomes. In view of the fact that a small amount of effusion was found at operation, this particular tumor region may be described as a semisolid malignant growth that represents an intermediate state in the development of peritoneal ascites. Another exceptional case was reported by Hsu (1954), who found a very wide range of chromosome polymorphism in a human sarcoma when it was cultured *in vitro*. All the 84 dividing cells were hyperdiploid. Not one contained the diploid number.

It has been mentioned previously that in the colony-forming ascites there are many intermediates between isolated single cells and large cell aggregates, which suggests that the latter must have been derived from the multiplication of one "parental" cell. This conclusion is supported by the fact that the size of the cell colony and the frequency of the various cell groups are related to the interval that has elapsed between successive paracenteses. It was found that the longer the interval, the higher is the incidence of cell colonies that contain large numbers of nuclei (80 to 100). It should be mentioned, however, that large cell colonies have been seen in effusions only a few days old. These must have been left over from the previous fluid. Another explanation for the presence of large cell colonies in these effusions may be that they are cast-off tissue fragments of metastatic adhesions (Baillif, 1954). Post-mortem examination of several cases with peritoneal effusions has shown solid infiltrations in the mesenteries, omenta, and inferior surface of the diaphragm (*cf.* Honigman, 1945). The metastatic deposits may represent foci of dissemination, from which isolated cells migrate and larger tissue fragments are shed into the effusion. Because of these possibilities, it is difficult to estimate the rate of cell multiplication in human ascites, especially in the colony-forming variants.

At present, we have no evidence to indicate the duration of life of malignant cells in the ascitic fluid. There is no doubt that their life is shorter than that of the cells in solid tumors. The life of the latter may be measured in months or years; that of the cells in the effusion, in days. Cell degeneration is going on continuously in human ascites, which usually contain a large number of dead cells. During the present investigation, dividing cells with excessive fragmentation of the chromosomes were seen in an effusion 21 days after radioactive colloid gold treatment. The type of injury indicates damage to the chromosomes that has occurred in the interphase nucleus, *i.e.*, the mitosis under observation was the first division since treatment 21 days previously. This finding seems to suggest that some free cells in ascitic fluid can live and remain active for a considerable time.

Discussion: The Origin of the Anaplastic Cell

Cytological analysis of human effusions produced evidence to show that the behavior of the malignant cells is greatly influenced by the conditions of the ascitic fluid. Thus it has been mentioned already that *the quantity and duration* of the effusion and *the rate* at which it has been produced are particularly important factors that can affect cell behavior. The depletion of nutrients and the accumulation of degradation products of cellular decay are believed to be responsible for the mitotic abnormalities that usually precede the mitosis-free period. The absence of dividing cells in the fluid indicates the reduction of nutritional conditions to a low level at which intracellular protein synthesis becomes impossible (Warburg and Hiepler 1952). Though these facts emphasize the importance of environment, the primary tumor has been found to be ultimately responsible for the histopathological nature and behavior of ascites.

It has been observed that the "free cell" effusions with a rather narrow range of cell polymorphism were associated with *anaplastic* primary tumors. On the other hand, the cell colony-forming ascites with a large spectrum of variation were found in the presence of *differentiated* primary tumors. It seems that anaplastic tumor cells exhibit a certain degree of stability in effusions, while cells of the differentiated malignant tissue show variability. The relatively higher degree of stability of the former cannot be attributed to adaptation. The evidence obtained in experimental ascites clearly indicates that this stability is the end product of mutation and selection (Klein 1953). These cells are assumed to have lost their tissue-specific enzyme complexes and, consequently, they represent a metabolically simplified or primitive system. Owing to the fact that the growth requirements of the anaplastic cell are less complex than those of the differentiated cell, the former can more easily be adapted to an existence in a fluid medium. This has been found to be the case in certain experimentally converted animal tumors (Klein 1955).

The behavior of the "differentiated" malignant cells in the primary tumor and in the ascitic fluid indicates that these cells are highly variable. Thus, it was found that, in "solid" tumor tissue, the frequencies of polyploid, multinucleate, degenerating, and abnormally dividing cells are higher in differentiated than in anaplastic malignant growth (Koller, unpublished data). Hauschka (1953) has emphasized that the morphological and physiological variants of the growing tumor indicate mutation and selection pressure exerted by the host. In ascites with less favorable and less stable environmental conditions than are present at the primary site, the variability or "plasticity" of the malignant cells is more clearly expressed. We found that in effusions associated with differentiated tumors, cellular independence is limited, cell colony-formation is of common occurrence, metabolic disturbances are frequent, and chromosome polymorphism has a wide range. Furthermore, our investigations have disclosed the mechanism by which aneuploid chromosome variation originates in the fluid medium. Abnormalities in spindle organization were found to be mainly responsible for irregular chromosome distribution. Nakahara (1953), studying the process by which chromosome variation arises in experimental ascites of rats, arrived at the same conclusion.

The fluid environment alone is not sufficient, however, to produce active cell variants with aneuploid chromosome numbers that can multiply. The capacity for such variations lies in the cells themselves.

In one of our patients, a pathological liver lesion was found in association with a benign peritoneal effusion. The ascites was of the "free cell" type and, in spite of the high number of degenerating cells present in the fluid that indicates extensive mitotic irregularities, chromosome polymorphism was not observed. The benign ascites contained several dividing cells, all with the diploid chromosome number. Kanô (1953) made a somewhat different observation on dividing monocytes of an experimental effusion in the rat.

Our investigation revealed that the ascitic fluid is a medium in which the cells of differentiated tumors undergo rigorous trial for fitness, adaptation, and survival. New biological variants appear in the cell population through mitotic disturbances and are exposed to natural selection. We observed that the evolutionary events lead toward dedifferentiation, *i.e.*, toward the development of the anaplastic cell. This particular cell type, by being less sensitive to nutritional disturbances, is better adapted to the conditions present in effusions and has a better chance to form adhesions on the serous membranes. The latter become important secondary foci from which cells migrate into the ascitic fluid and into more distant sites. Our studies clearly indicate that (1) the widespread dissemination of tumor that characterizes carcinomatosis in man is conditioned by the transformation of the differentiated cell into an anaplastic type, and that (2) this process is enhanced and greatly accelerated in the effusions. There is no doubt that, by the analysis of cell behavior in human ascites, further knowledge can be gained that will enable us to understand more clearly the biological forces and the way they interact during the development of malignant growth.

Summary

(1) Cytological analysis of 98 malignant effusions in man has shown that they are of two definite types with many intergrades: (1) in one, the cells behave as independent components; (2) in the other, they exhibit a capacity to form colonies with indistinct cell boundaries.

(2) Chromosome polymorphism (aneuploidy and polyploidy) is much greater in the latter type of effusion than in the free-cell ascites. The frequency of mitotic abnormalities is also higher in the cell colonies than in the free cells of ascitic fluid.

(3) Cell behavior in effusions is influenced: (1) by the volume of fluid in the serous cavities; (2) by the rate at which it has been produced; and (3) by the length of interval between paracenteses.

(4) Evidence has been obtained which suggests that adhesions on the serous membranes may act as secondary sites from which cells migrate into the ascitic fluid.

(5) Cell populations in double effusions exhibit similar behavior, which indicates the importance of the tumor at the primary site in determining the character of the ascites.

(6) After the administration of radioactive colloid gold (Au 198), cells with diploid chromosome number have the highest frequency, and the range of chromosome polymorphism is greatly reduced. The diploid class may be considered to be the "stem cells" in human effusions.

(7) Comparison of cell behavior at the primary sites and in effusions shows

that the anaplastic primaries are usually associated with "free-cell" ascites, and the differentiated tumors with cell colony-forming effusion.

Acknowledgment

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Discussion of the Paper

Doctor Cecilie Leuchtenberger (*Institute of Pathology, Western Reserve University, Cleveland, Ohio*): Doctor Koller is to be complimented for the fine contributions that he has made to the cytology of human carcinomatosis His extensive and careful analysis has not only helped to reveal the peculiarities and abnormalities of chromosomal behavior, but his data suggest a different approach to the study of the relationship between chromosomal numbers and DNA content in human tumors cells, a problem in which we have long been interested.

Using microspectrophotometry, which allows DNA analysis in individual cells, other investigators and we also have previously found that in normal tissues and in animal ascites tumors there is a good correlation between amounts of DNA and number of chromosomes. In other words, tissues containing cells with haploid, diploid, tetraploid, and octoploid numbers of chromosomes showed 1DNA, 2DNA, 4DNA, and 8DNA respectively. In an extensive study which we did on a variety of solid tumors,[1] we found that, in addition to the intermediate DNA values which would be expected because of mitosis, many tumor cells contained multiple DNA classes.

Stimulated by Doctor Koller's studies, we have also examined the malignant cells in a few ascitic fluids from human tumor cases.[2] While this ascites study is much too scanty to allow any generalization, nevertheless, the results closely resemble those of our studies in solid tumors. The human ascites tumors that we examined showed, without any exception, partly higher and partly multiple DNA values in single cells or agglomerations of cells with distinct cytoplasmic boundaries. These higher and partly multiple DNA values are in accordance with the DNA data that we found in rapidly growing solid human tumors. In some tumors, the basic DNA value was tetraploid instead of diploid.

Since Doctor Koller usually found the diploid number of chromosomes in nearly all solid tumors and in ascitic fluids containing cells with distinct cytoplasmic boundaries, the multiple DNA values found by our analysis can not longer be explained in each instance by multiple chromosomal numbers.

The question then arises whether some human tumors contain polytene chromosomes that would account for the multiple DNA values, or whether DNA multiples may occur in human tumor cells *without* any change in chromosomal status, that is, no change in the number of threads or of whole chromosomes. Whatever the answer will be, it now appears that, on the basis of Doctor Koller's chromosomal and our own DNA data, multiples of DNA values n human tumors are not necessarily synonymous with multiples of chromo-ome numbers.

A very rewarding result would no doubt be gained by a quantitative analysis f the DNA content in the material which Doctor Koller has so painstakingly nalyzed from the cytological and chromosomal point of view.

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FURTHER EVIDENCE FAVORING THE CONCEPT OF THE STEM CELL IN ASCITES TUMORS OF RATS*

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Zoological Institute, Hokkaido University, Sapporo, Japan

Cytological investigations based on morphological and statistical analysis of the chromosomes in several ascites tumors of rats conducted by the present author in collaboration with his co-workers have revealed the existence of the stem line (or stem lines) of tumor cells as primary contributors to the growth of the tumor. It has been shown that in each of the tumors studied, populations of tumor cells persist characterized by a high frequency of definite chromosome patterns specific to the kind of tumor differing from those of ordinary tissue cells (Makino and Kanô, 1951; Makino, 1952a, b; Makino and Kanô, 1953). The stem cells are persistent through serial transfers by dividing in a regular mitotic manner (Makino, 1952b; Makino and Nakahara, 1953a, b). They also remain unaltered in chromosome individuality through heteroplastic transplantations and through treatment with chemicals (Makino, 1952a; Makino and Tanaka, 1953a, b; Tanaka *et al.*, 1955). Since differences in the genetic constitution of these stem cells are correlated with differences in the genetic behavior of the tumor, it is the stem cells which determine the genetic behavior of a tumor. Further data to be presented in this paper supplement the evidence of the stem cells and strengthen the stem-cell hypothesis.

(1) *Double Inoculations with Two Different Tumors in the Same Host*

This article deals with cytological observations on the growth of two different ascites tumors of rats in the same host. Due to the presence of easily distinguishable characteristics, the MTK-sarcoma II and Takeda sarcoma were chosen as material. The MTK-sarcoma II (FIGURE 1) was established by application of azo dyes in a Wistar rat by Tanaka and Kanô (1951). The Takeda sarcoma (FIGURE 2) developed originally from the intraperitoneal transplantation of pieces of the crushed tissue of a spontaneous solid tumor of a white rat (Takeda *et al.*, 1952). These two ascites tumors are clearly distinguishable in many characters (Tonomura, 1954). The most pronounced difference lies in their chromosomes. The neoplastic population of the MTK-sarcoma II is provided with subdiploid stem cells with the modal number of 40 (FIGURES 4 and 5), while that of the Takeda sarcoma has cells with the tetraploid modal chromosome number of 84 (FIGURES 6 and 11). The average survival period of the host was found to be 9.2 days with the MTK-sarcoma II and 7.7 days with the Takeda sarcoma.

The experiments were made under the three schemes given below, along with control experiments. In every experiment, the tumor samples were obtained from the tumor-bearing rats on the fourth (sometimes the fifth) day

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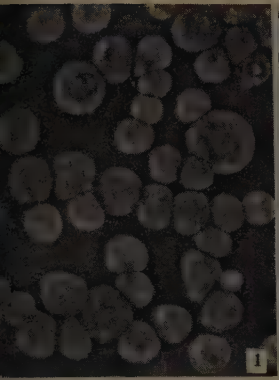
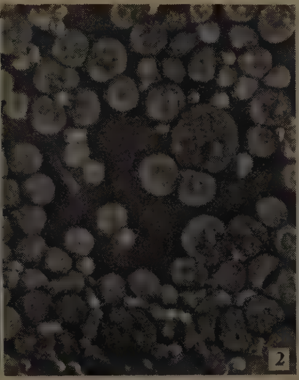




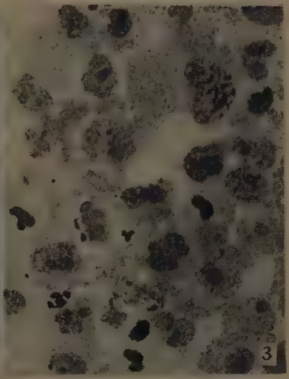


FIGURE 1. Tumor cells of MTK-sarcoma II, phase contrast. ×300
FIGURE 2. Tumor cells of Takeda-sarcoma, phase contrast. ×300
FIGURE 3. Damage of Takeda-sarcoma cells in the double inoculation with MTK-sarcoma II and Takeda sarcoma at 2-day interval. Acetic gentian-violet smear. ×400.

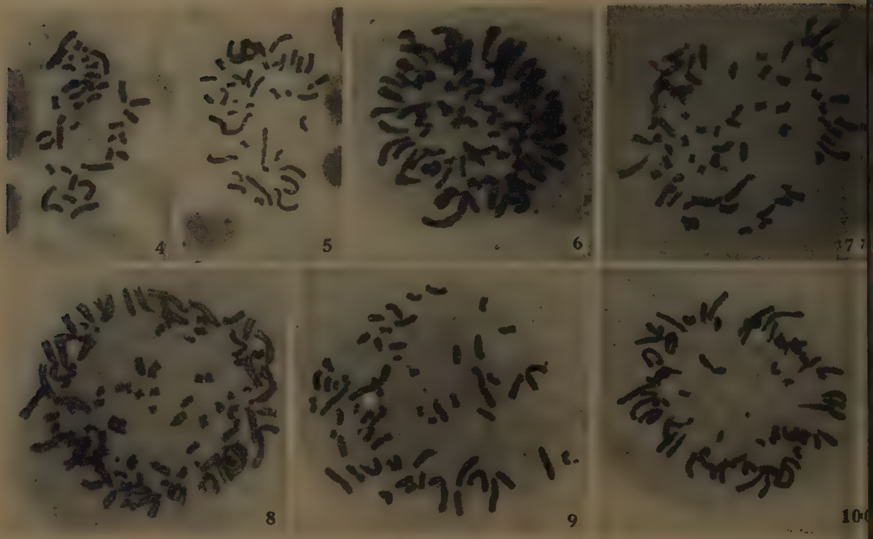
after transplantation in the MTK-sarcoma II, and on the third day in the Takeda sarcoma.

(a) *Simultaneous inoculation with the two tumors in the same host.* Samples from both tumors, about 0.05 cc. in volume, were injected simultaneously in the peritoneal cavities of the same host. After 24 hours after injection, multiplication of tumor cells derived from the two tumors had taken place in the same host. On the first day after injection, the frequency of the Takeda sarcoma cells was found higher than that of the MTK-II cells whereas, on the next day, the relative percentages of cells of both tumors were approximately similar. From the third day on, a gradual increase occurred in the number of the MTK-II cells. By the fifth day after inoculation, the Takeda sarcoma cells wholly disappeared so far as the metaphase cells were concerned.

The mitotic rate showed a sudden decrease on the third day after inoculation, when the Takeda sarcoma cells were found damaged. By this time, the ascites was considerably reduced. It seems probable that the decrease of the mitotic rate results from the reciprocal influence between the two kinds of tumor cells. Seven or eight days after injection, the MTK-II cells showed active division, manifesting a characteristic subdiploid chromosome pattern, followed by an accumulation of the ascites.

The above results indicate that the growth of the Takeda sarcoma is inhibited by coexistence of the MTK-sarcoma II in the same host.

(b) *Inoculation of the MTK-sarcoma II followed by the Takeda sarcoma.* The rats that received the MTK-sarcoma II, about 0.05 cc. in volume, were injected again two to four days later with the Takeda sarcoma, about 0.1 cc. in volume. When MTK-sarcoma II already existed, inoculation with the Takeda sarcoma induced a sudden decrease in the mitotic rate of tumor cells. The damage to the Takeda sarcoma cells occurring at this time was very striking, being followed by a reduction of the ascites (FIGURE 3). An increase in the mitotic rate of the MTK-II cells then followed with a gradual accumu-



FIGURES 4–10. Metaphasic chromosomes of tumor stem cells. Temporary smears stained with acetic dahlia ×800. FIGURES 4–5, MTK-sarcoma II. FIGURE 6, Takeda sarcoma. FIGURE 7, Usubuchi sarcoma. FIGURE 8 Watanabe ascites hepatoma. FIGURES 9–10, MTK-IV.

lation of the ascites in rats. Six to seven days after the second injection with the Takeda sarcoma, regrowth of the tumor had been attained in every rat through the multiplication of the MTK-II cells.

The evidence presented here shows that the growth of the Takeda sarcoma is completely inhibited by pre-existence of the MTK-sarcoma II in the same host.

(c) *Inoculation of the Takeda sarcoma followed by the MTK-sarcoma II* Two to four days following the injection of the Takeda sarcoma, about 0.1 cc in volume, the rats were inoculated with an approximately equal volume of the MTK-sarcoma II. Following the second injection with MTK-II, the mitotic rate of tumor cells showed a remarkable decrease. The tumor cells derived from both types of tumors seem to affect each other in the same host. There was great damage to tumor cells with consequent temporary regression of tumor growth. Most experimental animals survived from 11 to 16 days. Some of them showed regrowth of the MTK-sarcoma II, while others died of the Takeda sarcoma. Worthy of record are results from double inoculation at a two-day interval. The tumor growth was retarded for a long time without regrowth: six experimental animals survived from 57 to 70 days with the maintenance of tumor regression.

(d) *As controls*, double inoculations were undertaken either with the MTK-sarcoma II, or with the Takeda sarcoma, with the experimental procedures corresponding to the above experiments. In every test so undertaken, there was no significant influence on the growth of the tumor, with neither decrease of the mitotic rate nor damage to tumor cells.

Remarks. Graff *et al.* (1952) reported that sarcoma 180 inhibited the growth of carcinoma 755 in the same host when they were transplanted simultaneously, suggesting the possibility that sarcoma 180 elaborated a substance to which carcinoma 755 was sensitive. In the present material, the two types of tumor cells exist in direct contact with one another in the peritoneal cavity of the same host after inoculation. It seems highly probable that the inhibitory influence of one of the tumors on the other in the same host may be due mainly to the antagonistic action between the tumor cells of the two types of tumors. When the cellular activity of the one tumor overcomes that of the other, the former tumor may arrest multiplication of the tumor cells of the latter tumor. Under a balanced condition of cellular activity between the two types of tumor cells, the growth of both tumors may be retarded for a long period of time. Complete regression of the tumor, or complete recovery from the disease, has not been obtained in any experiment. In any case, the resistant tumor stem cells may persist alive under the inhibitory influence induced through double inoculation (for details, see Makino and Tonomura, 1955).

(2) *Experiments of Single-Cell Inoculation*

This article concerns experiments on inoculations of isolated single tumor cells followed by comparison of the chromosomes between single-cell clones and the stock tumor. The Hirosaki sarcoma, an ascites tumor of rats, was chosen as material because it is characterized by the presence of five stem-line cells—distinguishable from one another by 1, 2, 3, 4, and 5 V-shaped chromosomes of large size. At the time of experiment, the frequency distribution of the five type of stem cells appeared at 5.7, 40.3, 44.0, and 10.0 per cent for the 1V-, 2V-, 3V-, and 4V-type cells, respectively, with a disappearance of the 5V-type cell. Isolation of the single cells was made with the aid of a micromanipulator.

Positive results were obtained for single-cell inoculations in 4 of 51 trials in this study. The small percentage of positive results may be explained by supposing that most of the isolated cells were not viable, that they were lost or injured during the process of manipulation, or that they did not have the stem-cell's capacity to propagate autonomously. It is interesting that the four successful transplantations were all made in rats of a certain strain that was highly susceptible to the stock tumor. The tumors that developed in the four rats receiving single-cell inoculations gave rise to four distinct sublines capable of serial transfers. Cytological investigation of these four single-cell derivatives revealed that each tumor clone was characterized by a neoplastic population provided with tumor cells of a single-cell type. Two of the four sublines were found to be 3V-type cell derivatives, since they were characterized by populations of 3V-type cells, while the other two tumors were determined to be 2V-type cell derivatives because the populations consisted of 2V-type cells. It is evident that both 2V-type and 3V-type cells were more predominantly selected in isolation than the other kinds of stemcells on account of their prevalence in the stock tumor. The single-cell tumor clones isolated from the original tumor of a mixed-cell type showed a malignant growth, as well as characteristics of disease more or less comparable to the stock tumor. The variation of the chromosome number occurred within a narrower range in the single-cell clones than in the stock tumor. The frequent chromosome number modes for both cell types in the single-cell sublines were nearly identical with those in the stock line. The independent cytologica patterns of tumor cells have persisted as such for more than 70 and 40 seria transfers for the 2V-type cell and 3V-type cell clones, respectively.

Further cytological investigation was made on a tumor derived through a simultaneous inoculation with two different cells, one isolated from the 2V-type cell clone and the other from the 3V-type cell clone. It was revealed that this tumor contained two cell populations, one represented by the 2V-type cells and the other by the 3V-type cells.

Remarks. Thus far, isolation experiments with the Hirosaki sarcoma have resulted in the production of pure single-cell clones isolated from the origina stock tumor of a mixed-cell type. Each clone was characterized by a stemline chromosome pattern that is idiogrammatically distinct from those of the other clones and has persisted without visible shift during certain transplant generations.

All the cytological features derived from the isolation experiments in this study seem to have strengthened greatly the concept of stemline cells as progenitors of the neoplastic population (for details, see Makino and Kanô, 1955). The reality of the stem-cell hypothesis was strongly supported by Hauschka (1953a, b) on the basis of his results of single-cell inoculation experiments extensively conducted with several ascites tumors of mice. Recently, Kaziwara (1954) also produced results favoring the concept of stem cells after experiments of single-cell inoculation with the Ehrlich ascites carcinoma.

(3) *Persistence of the Stemline Chromosome Pattern in Subcutaneous Transplantation of the Ascites Tumor*

For the purpose of an examination of the persistence of the chromosome pattern of the stem cells, an experiment on subcutaneous transplantation of the ascites tumor was made with the MTK-sarcoma II (Tonomura, 1953). Generally, the ascites tumors of rats are transformed into a solid form when they are transplanted subcutaneously. Subcutaneous transplantations of MTK-sarcoma II were attempted with successful formation of solid tumors in many cases. The solid tumors thus formed were crushed with normal saline and injected into the peritoneal cavity of rats. This gave rise again to the ascites tumor. The chromosomes were investigated in the tumor cells of the ascites tumor thus produced and were compared with the chromosomes previously observed in the original ascites tumor before subcutaneous transplantation was undertaken. The results indicate that there is a complete similarity in the chromosome pattern of stem cells showing no slight shift between the ascites tumors under comparison. Every stem cell under study showed the subdiploid chromosome complex characteristic of the MTK-sarcoma II. It was thus shown that the chromosome constancy had persisted after the transformation of the ascites tumor into a solid tumor.

Remarks. A similar feature of chromosome persistence in the tumor stem cells was observed also in the experiments on heteroplastic transplantation Makino 1952a, b). The malignant growth of the Yoshida sarcoma is highly host-specific to a certain strain of white rats (*Rattus norvegicus*). The Yoshida sarcoma cells transplanted into related animals such as black rats (*Rattus rattus*), white mice (*Mus musculus*), field mice (*Apodemus geisha*), voles (*Clethrionomys bedfordiae*), and guinea pig (*Cavia cobaya*) survived for a short length of time with a few mitotic figures. Cytological observations of these mitotic figures revealed that the chromosomes of the Yoshida sarcoma cells remained unaltered in cases when they were transplanted into heterogeneous hosts.

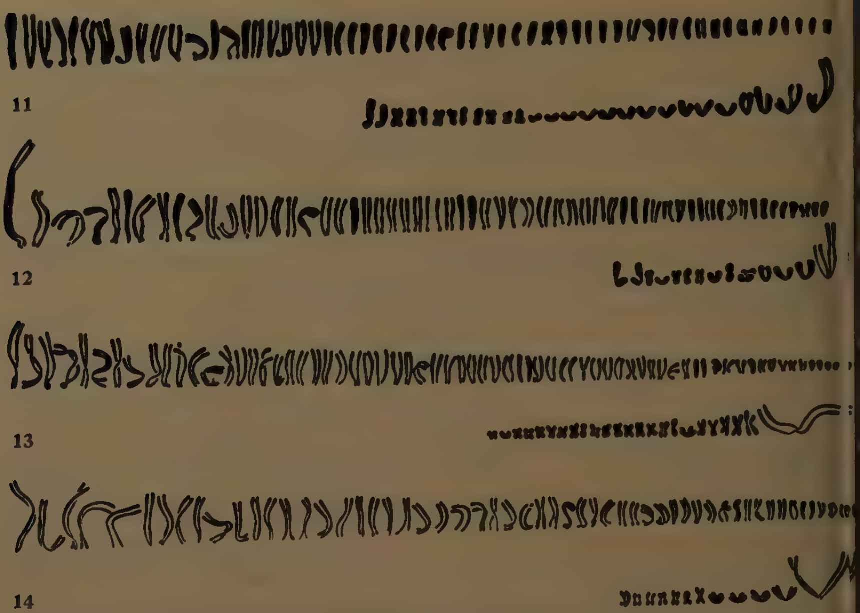
(4) *Polyploid Ascites Tumors in Rats*

It has been shown by previous investigations (Makino and Yoshida, 1951; Makino, 1952a, b; Makino and Kanô, 1953; Umetani, 1953) that the Yoshida sarcoma, MTK-sarcomas I, II, and III, and Hirosaki sarcoma are all ascites tumors of white rats characterized by neoplastic cell populations of a subdiploid (near-diploid) nature. In each of these tumors there is a neoplastic population (or populations) of tumor cells provided with well-balanced subdiploid complexes of chromosomes, 40 or thereabouts in number, characterized by stem-line chromosome patterns specific to the type of tumor. Furthermore, there have been found two population types of stem cells distinguishable by their chromosome complexes in the MTK-sarcoma I, and five types of such cells in the Hirosaki sarcoma (Makino and Kanô, 1953). These neoplastic cell populations possess different chromosome patterns specific to each, and they differ also in their mitotic activity.

Very recently, several ascites tumors characterized by neoplastic populations of polyploid stem cells have been shown to occur in certain rat strains. They are the Takeda sarcoma, Usubuchi sarcoma, Watanabe ascites hepatoma, and MTK-IV, all developing in the peritoneal cavity of white rats in the form of an ascites tumor. The first three are subtetraploid tumors, for their predominant stem-cell populations are characterized by a near-tetraploid chromosome complex, while the MTK-IV is seen to be a subtriploid tumor since the predominant neoplastic population is represented by near-triploid cells.

The Takeda sarcoma. The Takeda sarcoma shows a wide variation in chromosome number ranging from 37 to 163, but the modal values lie at the range from 79 to 90. The most frequent number is 84, just the $4n$-number of rats (Tonomura 1954). Morphologically, the complement shows characteristic numbers of rod-, V-shaped, and J-shaped elements of varying sizes (FIGURES 6 and 11). The occurrence of a prominent J-shaped chromosome of outstandingly large size, with frequent occurrence of a large V-shaped element, one or sometimes two in number, is very remarkable in the complex. Thus subtetraploid cells differ in the shape of some of their chromosomes from the chromosomes of normal tissues. It is interesting to find that the subtetraploid complex of the Takeda sarcoma cells seems not to be a simple euploid multiple of the basic complex of the rat.

The Usubuchi sarcoma. The Usubuchi sarcoma was originally produced by



FIGURES 11–14. Serial alignments of chromosomes of tumor stem cells. FIGURE 11, Takeda sarcoma. FI URE 12, Usubuchi sarcoma. FIGURE 13, Watanabe ascites hepatoma. FIGURE 14, MTK-IV.

Usubuchi *et al.* (1953) by peritoneal injections of crushed pieces of the tissu of a tumor developed in a white rat by applying methylcholanthrene. Cyto logical investigation has made it clear that this ascites sarcoma is to be classi fied as a hypotetraploid line, since its neoplastic population is characterized b predominant hypotetraploid stem cells. The modal variation of the chromo some number in stem cells was found to range from 65 to 80, the most frequen numbers falling between 70 and 76. The ideogram contains characteristi numbers of rod-, V-shaped, and J-shaped chromosomes and is clearly dis tinguishable from that of the Takeda sarcoma through difference in numbe and shape of the composing elements (FIGURES 7 and 12). It is again note worthy here that the hypotetraploid complex of the Usubuchi sarcoma cell does not represent a simple duplication of the basic somatic complex of rats.

The Watanabe ascites hepatoma. The Watanabe ascites hepatoma wa originated from a hepatoma developed in one of several rats by repeated ap plication of hot water (72° C.) in their peritoneal cavities. A transplantabl ascited tumor was induced by injecting in the peritoneal cavity of the ra crushed pieces of tissue of the hepatoma thus formed (Watanabe and Mat sunaga, 1954). Cytological observation has established that the Watanab ascites hepatoma is a type of tumor of mixed stem-cell lines, since this tumo is represented by at least three different neoplastic populations provided wit subtetraploid, subdiploid, and subhexaploid stem cells, respectively. Amon the three populations, the subtetraploid population is predominant in occur rence, as well as in mitotic activity. In the samples so far examined, approxi mately 50 per cent of the cells were subtetraploid; 30 per cent, subdiploid cells and about 13 per cent, subhexaploid cells, with a few per cent of aberrant cells. The modal variation of the chromosome number was found to be 80 to 90 for the subtetraploid cells, 40 to 44 for the subdiploid cells, and 116 to 125 for the subhexaploid cells. The chromosome constitutions are highly specific to the three cell types, the number of the rod-, V-shaped, and J-shaped elements forming the complement being characteristic for each type. The subtetraploid tumor cells differ in the shape of some of their chromosomes from subdiploid tumor cells, as well as from normal tissue cells (FIGURES 8 and 13). Though detailed morphological analysis of the chromosomes has not been completed as yet, it seems highly probable that the polyploid complexes here concerned are not simple euploid multiples of the basic complex. For instance, the subtetraploid cell seems not to be a simple duplication of the diploid cell. It appears also that the subtetraploid and subdiploid cells are not transitional between them. Furthermore, there is evidence that a correlative change of the cellular activity takes place in the three-cell types. A preliminary study of the frequency distribution of the metaplasic tumor cells made through a transplant generation has revealed that when the subtetraploid cells are high in frequency, the subdiploid cells appear at a low frequency.

The Watanabe ascites hepatoma is also remarkable in containing a number of giant tumor cells that seem to be derivatives, or by-products, of certain stem-line cells. These cells seem to contain irregular chromosome constitutions with aberrant chromosome numbers. Usually they are multipolar in division. FIGURE 15, A to F, shows the successive series of a division process followed through a single, probably subtetraploid stem cell, showing a regular mitotic division, while FIGURE 16, A to F, indicates the successive series of a tripolar division taking place in an aberrant cells, probably irregular in its chromosome constitution. Evidently such cells are no longer able to divide, since they have lost some of their capacity for normal reproduction such as the ability to form functional spindles. Furthermore, these aberrant cells may be disturbed in their chromosome combination, which is essential for gene survival, whereas the subpolyploid stem cells, as progenitors of the neoplastic population, have retained the chromosome combination capable of normal mitotic reproduction.

MTK-IV. This is also one of the transplantable ascites tumors of rats, artificially induced by application of azo dye. The type of this tumor—sarcoma or carcinoma—has remained undetermined as yet. It is striking, in contrast to the other tumors so far described, that this tumor is characterized by a neoplastic population in which subtriploid stemline cells are highly predominant. In the samples so far examined, over 85 per cent of the dividing neoplastic cells were subtriploid. In addition, a few subdiploid cells* appeared, together with some aberrant cells. The modal variation of the chromosome number of the subtriploid cell falls between 62 and 68, the most frequent number being 67. On the basis of the chromosome number it can be said that this is the tumor of a hypertriploid line.

The subtriploid stem-cells are also very characteristic in their chromosome

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* In the present status of investigation, it is questionable whether these subdiploid cells are neoplastic cells or monocytes.

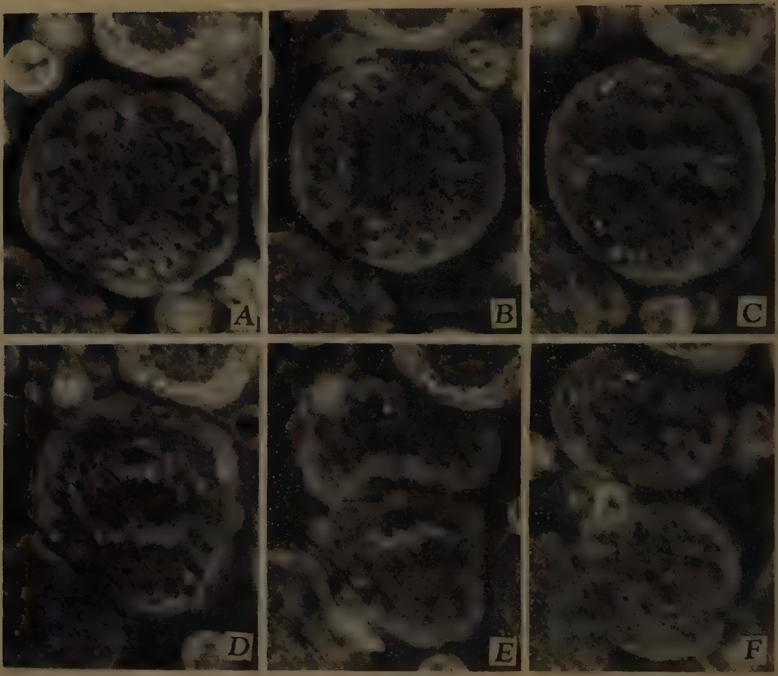

Figure 15, *A–F*. Successive stages of a regular mitosis followed in a single tumor cell of Watanabe ascit hepatoma. Phase contrast, ×1000. *A–B*, prophase (18 min.). *C*, metaphase (30 min.). *D*, anaphase (7 min. *E–F*, telophase (34 min.).

complex, having a specific combination of rod-shaped, V-shaped, and J-shape elements, among them a V-shaped one of large size being prominent (FIGURE 9 and 10 and FIGURE 14). Although the analysis is incomplete, it is probabl that the subtriploid chromosome complex here concerned is not a mere tripl combination of a basic complex of the rat and, furthermore, that some of th chromosomes in these cells differ in shape from the chromosomes of norma tissue cells. The subtriploid cells are generally 20 to 21 microns in diameter Their mitotic process was studied in a temporary hanging-drop culture followed through a single cell, resulting in a confirmation of regular mitotic behavior. Figure 17, A to H, illustrates the successive stages of mitosis followe in a single subtriploid cell.

Remarks. It has been shown in the foregoing descriptions that, in addition to the subdiploid lines of ascites tumors of rats already reported, subtetraploi and subtriploid lines also occur. Each line is characterized by a stem-cel lineage (or lineages) with near-tetraploid or near-triploid chromosome constitutions, specific to the different types of tumor. In the Takeda sarcoma, th Usubuchi sarcoma, and the Watanabe ascites hepatoma, the predominan near-tetraploid populations are the primary contributors of neoplastic growth while the MTK-IV is characterized by a neoplastic population represented by predominant hypertriploid tumor cells. Reviewing the results of the presen

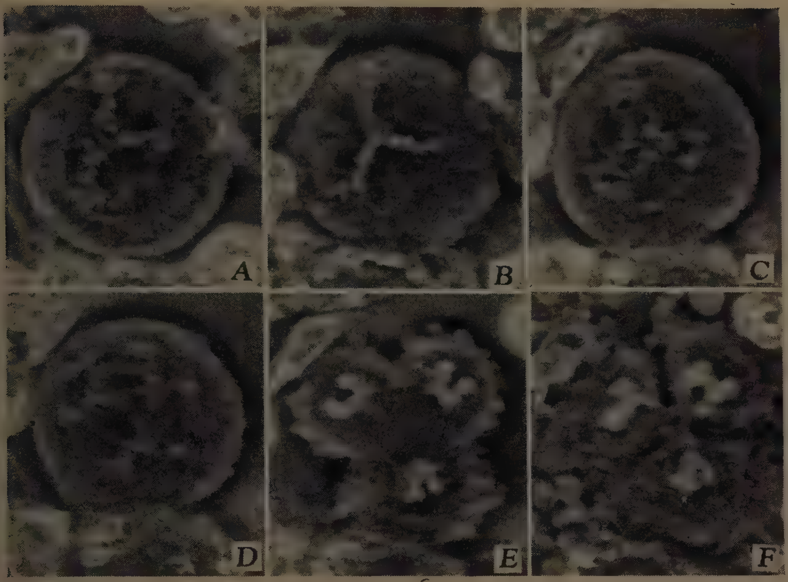

FIGURE 16, *A–F*. Successive stages of a tripolar mitosis followed in a single tumor cell of Watanabe ascites hepatoma. Phase contrast, ×1000. *A–B*, metaphase (47 min.). *C–D*, anaphase (6 min.). *E–F*, telophase (19 min.).

study, it becomes evident that the populations of cells partaking in the neoplastic growth are connected with the euploid relation of the chromosomes between the tumors under study, namely, the neoplastic cells possessing the capacity to reproduce in each tumor exist in near-euploid relationship, as in the 2*n*-, 3*n*-, and 4*n*-relations. In this connection, it is interesting to know that the ordinary tissue cells taking important part in growth also show a simpler relationship. In the cytological study of the regenerating rat liver, Makino and Tanaka (1953c) and Tanaka (1953) have found that, although there is a wide variation of the chromosome numbers in the liver cells of adult rats during restoration after partial extirpation, the most frequent chromosome numbers fall into the following three ranges: the diploid range (36 to 46 chromosomes), the triploid range (59 to 68 chromosomes), and the tetraploid range (78 to 89 chromosomes). The cells of the diploid range exist always in the highest frequency, and they then play a significant role in the regenerating rat liver, with cooperation of the cells of tetraploid and triploid ranges, which appear in more reduced percentage than the former. In light of the above facts, it seems probable that in mammalian tissues, either normal or neoplastic, the cells provided with euploid (or near-euploid) chromosome combination seem to possess a greater reproductive capacity than the aneuploid cells having aberrant chromosome constitutions, and that they play an important role in the growth of tissue. Probably these cells have well retained the gene combination necessary for reproductive activity. The results of Hsu and Pomerat (1953) and Sachs (1954) on the mammalian somatic chromosomes seem to favor this view.

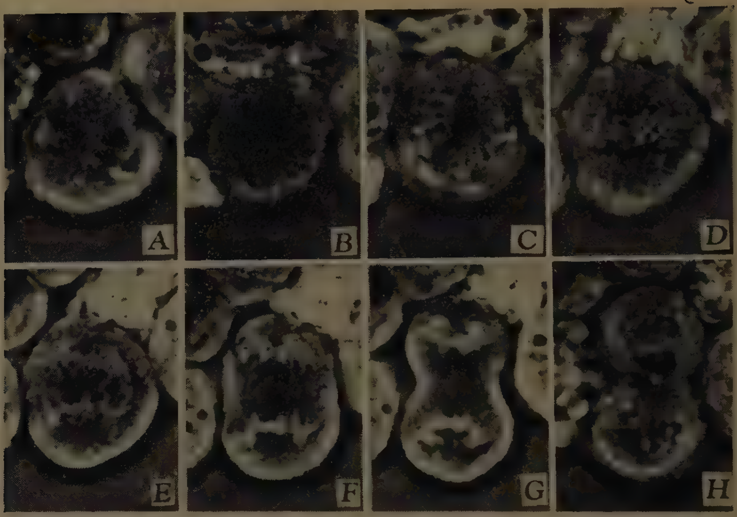

FIGURE 17, *A–H*. Successive stages of a regular mitosis followed in a single tumor cell of MTK-IV. Pha contrast, ×1000. *A*, resting stage. *B*, prophase (10 min.). *C*, metaphase (31 min.). *D–F*, anaphase (6 min. *G–H*, telophase (20 min.).

Evidence presented in this study indicates that, in both subtetraploid an subtriploid tumor cells, the chromosome complexes do not involve a simpl euploid multiple of the basic somatic complex of rats. Furthermore, th chromosome patterns of these tumor cells differ in the different types of tumors being highly specific to each tumor type. From the above facts, the followin speculation may be possible in relation to the origin of these tumor cells: tha probably the mutational change in the chromosomes might have taken plac in certain tissue cells under their already duplicated state, or after the cell had duplicated into tetraploid or triploid forms.

Summary

In this paper, additional evidence is presented that favors and strengthen the concept of stemline cells as progenitors of the neoplastic population. I each tumor so far studied, there is a neoplastic population (or populations the tumor cells carrying a characteristic stemline chromosome pattern (o patterns). These cells are capable of regular mitosis and are thus persisten in their genetic constitution through transfer generations. They serve a primary contributors to the growth of the tumor. It is these stemcells tha maintain the distinct pattern of each tumor and determine the genetic behavio of a tumor.

Note. After this paper went to press, the author found evidence that the subtriploi tumor cells of the MTK-IV were induced from hypotetraploid cells through elimination c certain numbers of chromosomes.

Acknowledgment

The author acknowledges gratefully that the studies reported in this paper were conducted in collaboration with Doctors F. Watanabe, T. Tanaka, K. Kanô, H. Nakahara, A. Tonomura, T. A. Okada, Y. H. Nakanishi, and S. H. Hori.

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CHANGES IN NUCLEAR DNA IN NORMAL AND ASCITES TUMOR CELLS

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It is a commonplace that the study of the nature and function of genes is fundamental in biology, and it is natural that we should give considerable attention to desoxyribose nucleic acid (DNA), whose close association with the chromosomes has been supported by much of the available evidence. Moreover, DNA can be isolated pure, and its chemical and physical properties in certain states are now fairly well understood. Interest in DNA has been further accentuated because it is one of the very few substances whose quantity can be measured in individual cells.

Two optical methods are suitable for this quantitative measurement of DNA. In the first, we can make use of the natural absorption at 260 mμ due to the purines and pyrimidines of the nucleic acid molecule and, in the second, of the absorption at 550 mμ of the Feulgen DNA complex. The former requires a complicated ultraviolet microspectrophotometer and is not specific for DNA, but is perhaps most useful in combination with other methods in studying the relationship of DNA to other cellular components. On the other hand, instruments for measuring Feulgen stain to various degrees of accuracy are generally available and have been extensively used for many problems.

Before presenting our own results on the quantitative behavior of DNA in both normal cells and those of some ascites tumors of the mouse, we shall consider very briefly some of the views on the role of this component in the cell and then discuss at greater length the factors that cause variation in the measured amount of DNA in individual nuclei, as an aid to extending the usefulness of these comparatively new methods of quantitative measurement in cell biology.

The two main opinions concerning the function of DNA may be simplified as follows: on the one hand, it is thought that the units that maintain genetic continuity are of a DNA-protein nature and that, therefore, all cells whose characteristics are transmitted from one cell generation to the next should contain an amount of DNA depending, within a given species, on the number of chromosomes present. In addition, the amount of DNA must at some stage double if the cells are to divide and still maintain this basic quantity.

On the other hand, it has been suggested that proteins may constitute the units that maintain genetic continuity and that DNA has some subsidiary function.

Experimentally, this second and less orthodox hypothesis is supported by any variation from the standard haploid amount of DNA that is not accounted for by (a) variation in chromosome number, (b) synthesis of DNA in preparation for division, or (c) errors in measurement. However, it has been an interesting characteristic of the history of DNA measurements, from the original biochemical determinations on counted nuclei by Boivin, Vendrely, and Vendrely[1] and by Ris and Mirsky[2] up to the present time, that improved methods have shown that most of the reported discrepancies were due to one or other of these three causes. There remain, however, certain notable exceptions, such as the echinoderm oöcytes investigated by Marshak[3] with an isotope dilution method, and the so-called empty nuclei of the bean root by Chayen[4] that apparently contains no DNA. To these examples may be added the smaller variations in DNA content following cold treatment that have been observed by cytogeneticists[5] and the measured variations due to chemical treatments, such as the application of croton oil and alloxan.[6, 7] All these require further investigation.

(*1*) *Sources of Variation in Measured Amounts of DNA*

(a) *Chromosome number.* In normal cells, except those where polyploid series occur, the variation of chromosome number about the diploid may not be very large. Thus Hsu and Pomerat[8] have reported that in normal cells of the rat the distribution of chromosome number about the mean value was ±7 chromosomes in 42. In the past, a similar variation in the Feulgen stain would not have been reliably demonstrated by photometric methods. On the other hand, the chromosome number is known to vary considerably in a variety of animal and in a few human tumors. In these tissues, the average DNA content per cell as measured biochemically shows a variation similar to that obtained by chromosome counting procedures, as was strikingly demonstrated by Klein[9] and by Hauschka and Levan[10] for various ascites tumors of the mouse. The variation of DNA between individual cells in tumors has also been studied by both the ultraviolet and Feulgen methods, notably by Leuchtenberger, Klein, and Klein[11] in ascites tumor, and by Leuchtenberger, Leuchtenberger, and Davis[12] for a variety of human tumors. While the work has often shown a much wider range in the amount of DNA per nucleus in the cells of tumors than in normal tissues, it is difficult to determine whether this is due to a variation in chromosome number or to the doubling of DNA consequent on an increased rate of division.

(b) *The doubling of DNA.* As we have already stated, DNA constancy must be maintained by DNA doubling, but the period during which this increase occurs may vary in different species or in different tissues of the same species. It is now fairly certain, however, that it normally proceeds during interphase, usually near the end of this period and before the onset of mitosis. This has been shown in two ways. The first method was used by Swift,[13] who found that in a population of dividing cells with the same number of chromosomes the prophases had twice as much DNA as the telophase nuclei and that the interphase cells had amounts of DNA ranging between these two values. It was, therefore, concluded that this interphase range reflected the doubling of DNA during interphase. A second method, which we have used, is applicable to cells that can be photographed for a long period prior to measurement. It consists in filming a sector of a tissue culture with low-power phase contrast. Measurements of DNA are made on cells identified in the film, which can then be used to ascertain the time elapsed between mitosis and measurement.[14]

In this way, we can measure directly not only the time in interphase when synthesis begins and the length of the period of synthesis, but also the interphase and mitotic times, and so obtain a fairly complete picture of the cell's behavior. Owing, however, to the few types of material available that are suitable for this method, we have investigated the conditions under which a random sample of measurements on interphase cells may be used to calculate the fraction of interphase during which the doubling of DNA or other substances occurs.[15] These conditions are as follows: first, the variation in chromosome number should be small; second, the growth characteristic of the cell population must be known (this is often logarithmic, as in the case when every cell eventually divides); third, the sample should be representative not only of all the cells but also of all stages of synthesis; and, fourth, the quantity of substance synthesized should be the same for all cells after any *fraction* of the interphase time which, however, may itself vary in length.

The first condition regarding chromosome number does not necessarily hold for tumor cells and will be discussed further in connection with the experimental results. The second condition holds for many actively dividing tissues and is closely associated with the third, which means, in effect, that a correction is required for any large proportion of cells that have very long interphase periods. The last condition is the most difficult to control, but it indicates that we cannot expect more than an average synthesis curve from this method. When it has been possible to compare the results of the sampling and of direct observation, however, quite good agreement is obtained.

This analysis is particularly applicable to normal cells grown in culture and to tumor cells in the ascitic form, since variations in the cellular environment may be expected to be small. It is in the cells of normal and of neoplastic tissues, however, that these methods should prove most useful eventually, since it is possible to distinguish different cell types under the microscope. Unfortunately, however, the difficulties are much greater due to our almost complete lack of information about interphase and mitotic times, rates of growth, and cell replacement.

Most tissues have a low mitotic index, and the mitoses found may be confined at any one time to certain areas, or be subject to marked rhythms. This low mitotic index may be correlated either with a state in which most of the cells have very long interphase and short mitotic periods, as probably in the later stages of embryonic growth, or with a condition in which a few cells are dividing rapidly while the remainder do not multiply, as in adult tissues or the early stages of malignancy. These factors may well explain why a spread of DNA values, presumably evidence for DNA synthesis, is sometimes obtained from tissue sections with a very low mitotic index. On the other hand, DNA synthesis that is not followed by mitosis is found in the elongating zone behind the bean root meristem.[16]

The effect of DNA doubling on the spread of values caused by a range of chromosome numbers obviously can be eliminated if we can confine measurements to a well-defined stage of the cell cycle, such as metaphase. Measurements on mitotic stages are inaccurate, however, unless special methods such as those outlined in the next section are designed to overcome the difficulties.

(c) *Feulgen microphotometry—cell scanning and cell crushing.* The quantitative application of the *Feulgen reaction* as being specific for DNA has often been criticized, but it now appears that, provided its limitations are appreciated,[17] accurate and reproducible results may be obtained. As Leuchtenberger[18] has pointed out, the extensive use of this method has been justified by the good agreement between the results obtained with this technique and by measurement of ultraviolet absorption, biochemical analysis, and chromosome counting. There remain, however, certain physical limitations to microphotometric methods that are independent of the staining procedure. These limitations and the methods designed to overcome them are relevant to the validity of our results and must be considered further.

Many living cell nuclei are optically homogeneous but, after fixation and staining, the absorbing material may be aggregated into small discrete particles and, in addition, parts of the specimen will often be out of focus. With many photometric methods, these conditions cause errors that become very serious if the measurement of metaphase chromosomes is attempted.[19] One way of reducing them, developed by Ornstein[20] and by Patau,[21] is known as the two-wave-length method but, in this laboratory, we have tried a somewhat different approach that we believe to be quicker and easier. We have employed a new instrument for stain measuring designed by Deeley,[22] in conjunction with a method of controlled crushing of the specimen with a special condenser described by Davies, Wilkins, and Boddy.[23] Deeley's instrument (FIGURE 1) consists of a microscope that projects the image of a small area including the specimen, defined by a diaphragm, on to an aperture at the image plane. This aperture is mechanically scanned so that light is allowed to reach the photomultiplier from only a fraction of the specimen small enough almost to eliminate errors due to inhomogeneity. The photomultiplier, which has a special circuit associated with it, produces consecutively a signal proportional to the extinction of each small element of the specimen, and these signals are then integrated and read off on a meter. Measurements of the same field with and without the object give, by difference, the total amount of stain present. Each reading requires three seconds and, normally, several are taken from both object and clear field. In this way, the stain in a large number of nuclei can be measured without any need for determining the area or shape of the structures as in most of the other methods commonly used.

This scanning device reduces errors due to inhomogeneity, but there remain inaccuracies due to the high extinctions (>1.0) of many objects and to parts of the specimen being out of focus. These may be virtually eliminated with a "crushing" condenser, which is an ordinary microscope condenser with a hemispherical top component that can be brought up to flatten the specimen between a sheet of cellophane and the coverslip. This simple device flattens nuclei or chromosomes and reduces their extinction to a value within the range of reliable measurement. It is desirable to use a nonhardening fixative during preparation, since hard objects tend to fragment under the treatment but,

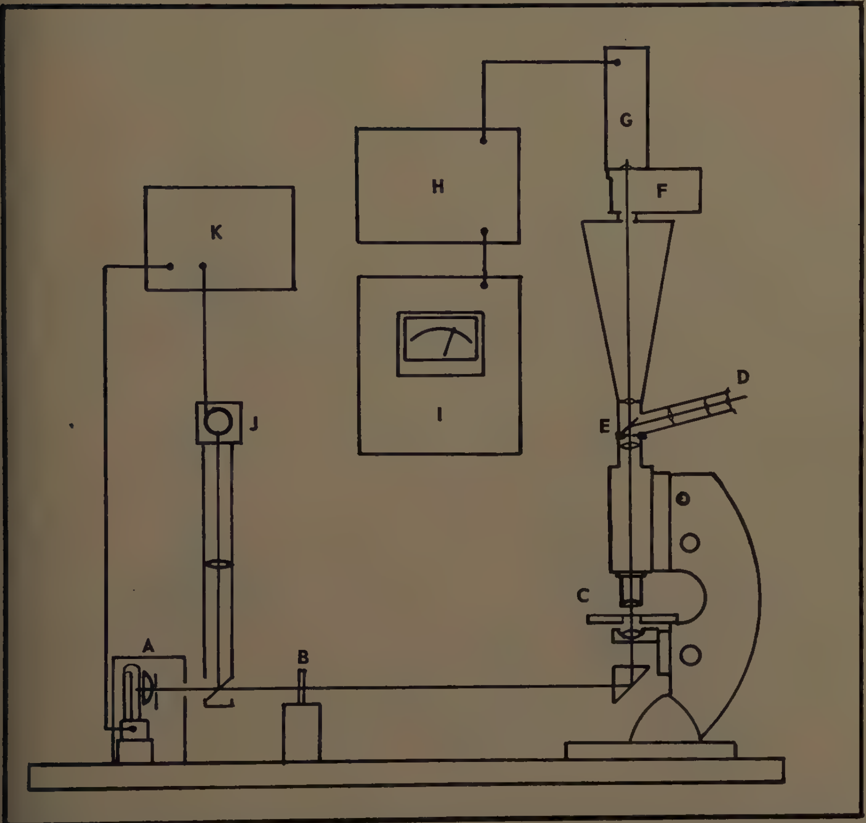

FIGURE 1. The rapid scanning photoelectric instrument built by Deeley. (A) Mercury arc light source; (B) Wratten 58 and 77A filters to isolate mercury green light; (C) microscope; (D) viewing eyepiece; (E) moveable mirror; (F) scanning aperture; (G) photomultiplier; (H) logarithmic amplifier; (I) integrator and meter; (J) and (K) photomultiplier and current amplifier of mercury arc stabilizing circuit.

normally, a combination of the "crushing" and the shearing motions of the condenser is successful. Thus after suitable fixation, cell nuclei may be flattened in a controlled manner while still retaining their basic morphological appearance and, especially important, without affecting adjacent nuclei. There is therefore no danger of mistaking identity in a heterogeneous population. The crushing operation is rapid and does not add materially to the time of measurement.

Two other factors have influenced the comparatively high accuracy which we believe characterizes the results now to be presented. Measurements have only been compared and illustrated in the same figure from the same microscope slide and we have only measured whole cells either grown in culture or obtained by a smearing technique.

(2) *Chromosome Counting and DNA Values*

As previously mentioned (see also Richards[24]), an accurate measurement of DNA Feulgen in stages of the cell cycle when synthesis is not in progress is

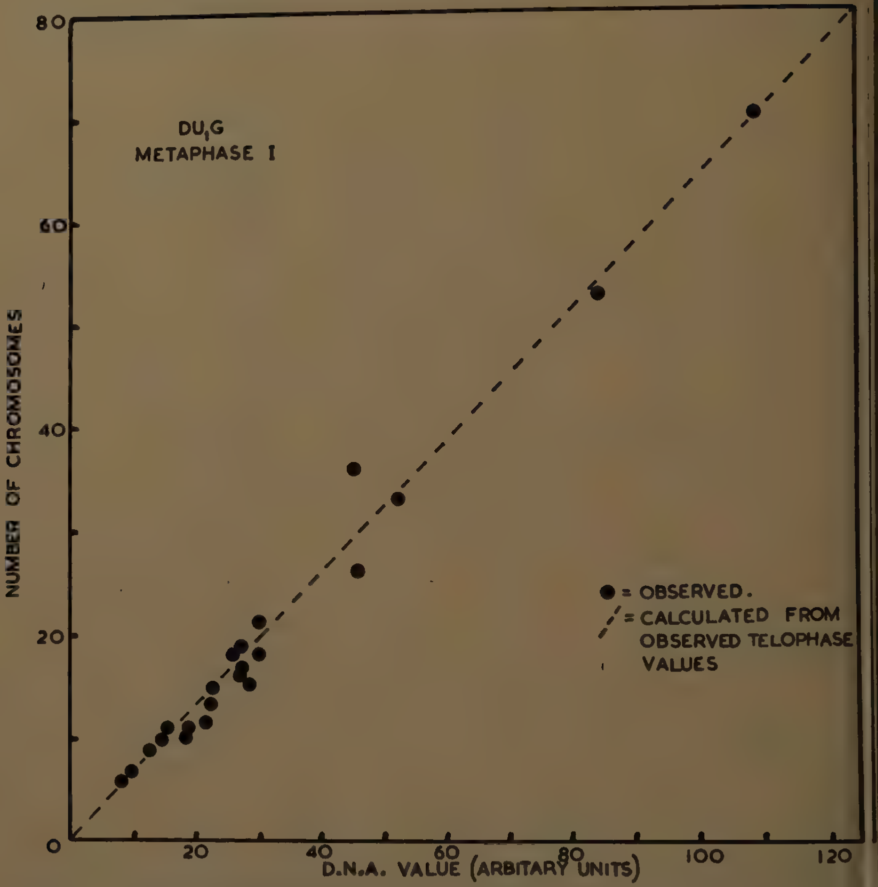

FIGURE 2. Plot of DNA amount against number of chromosomes in Krebs ascites tumor. Broken line connects the origin with the mean DNA value (125) of a large telophase sample, assuming that these contain 8 chromosomes.

proportional to the chromosome number. FIGURE 2 shows a plot of DNA values against the number of metaphase chromosomes of the Krebs 2 ascites tumor. The curve has been obtained by including within the measured area different groups containing a varying number of chromosomes. The correlation obtained is better with the larger number of chromosomes owing partly to a reduction in instrumental errors, but partly also, as might be expected, to the decreasing influence of variations in chromosome size. From this point of view, the larger number of small chromosomes in ascites tumors makes them particularly suitable material for this kind of measurement.

In FIGURE 3 are compared the distributions of metaphase chromosome numbers in the Ehrlich and Krebs ascites tumors determined by counting methods with those obtained from metaphase DNA values of the same or similar tumor samples. FIGURE 3 shows (AI) a histogram of chromosome numbers in the

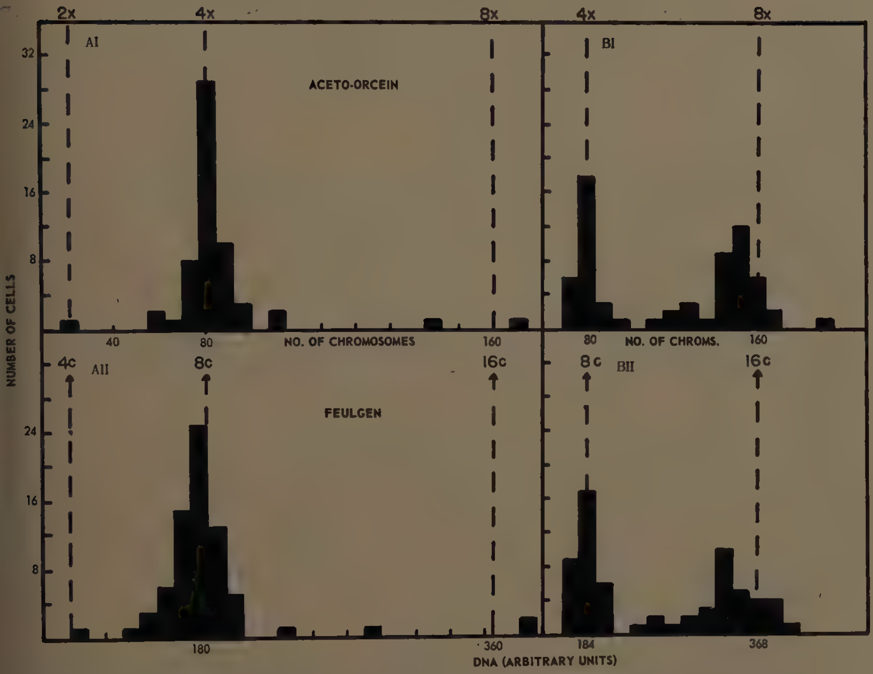

FIGURE 3. Comparison of chromosome counts obtained by the aceto-orcein squash method and the DNA estimation on metaphase groups:
Ehrlich ascites tumor: (AI) Aceto-orcein squash (Levan and Hauschka, 1953); (AII) DNA estimation.
Krebs ascites tumor—colchicine-treated: (BI) Aceto-orcein squash; (BII) DNA estimation.

Ehrlich tumor published by Levan and Hauschka[25] together with (AII) a distribution of metaphase DNA values in a sample of the same tumor plotted on a comparable scale. A similar comparison (BI-II) is shown for the Krebs tumor which has been treated with colchicine. The basic chromosome number is 80 in the tetraploid Ehrlich and Krebs tumors, which corresponds to the 4c value at telophase and the 8c at metaphase, where c represents the amount of DNA in a mouse sperm. Most tumor populations also contain a variable number of metaphases with twice the normal number of chromosomes (2s) whose DNA value will be 16c. It sometimes happens that, owing to the limited area available at the scanning aperture, the 16c (2s) metaphases, after crushing, are too large to measure. This selective effect may account for the discrepancy between the results of chromosome counting and of DNA estimation, for in the colchicine-treated sample, the proportion of 2s metaphases is artificially increased due to colchicine causing the "doubling-up" of many of the earlier blocked *s* metaphases. Nevertheless, the peak obtained for 2s metaphases by the DNA extinction method may be seen clearly. It is interesting to note that no numbers much below *s* were obtained in the colchicine-treated tumor sample, and this will be re-examined later.

While there may be a slight tendency to select smaller cells with the DNA method, it is possible, however, to make DNA measurements on abnormal

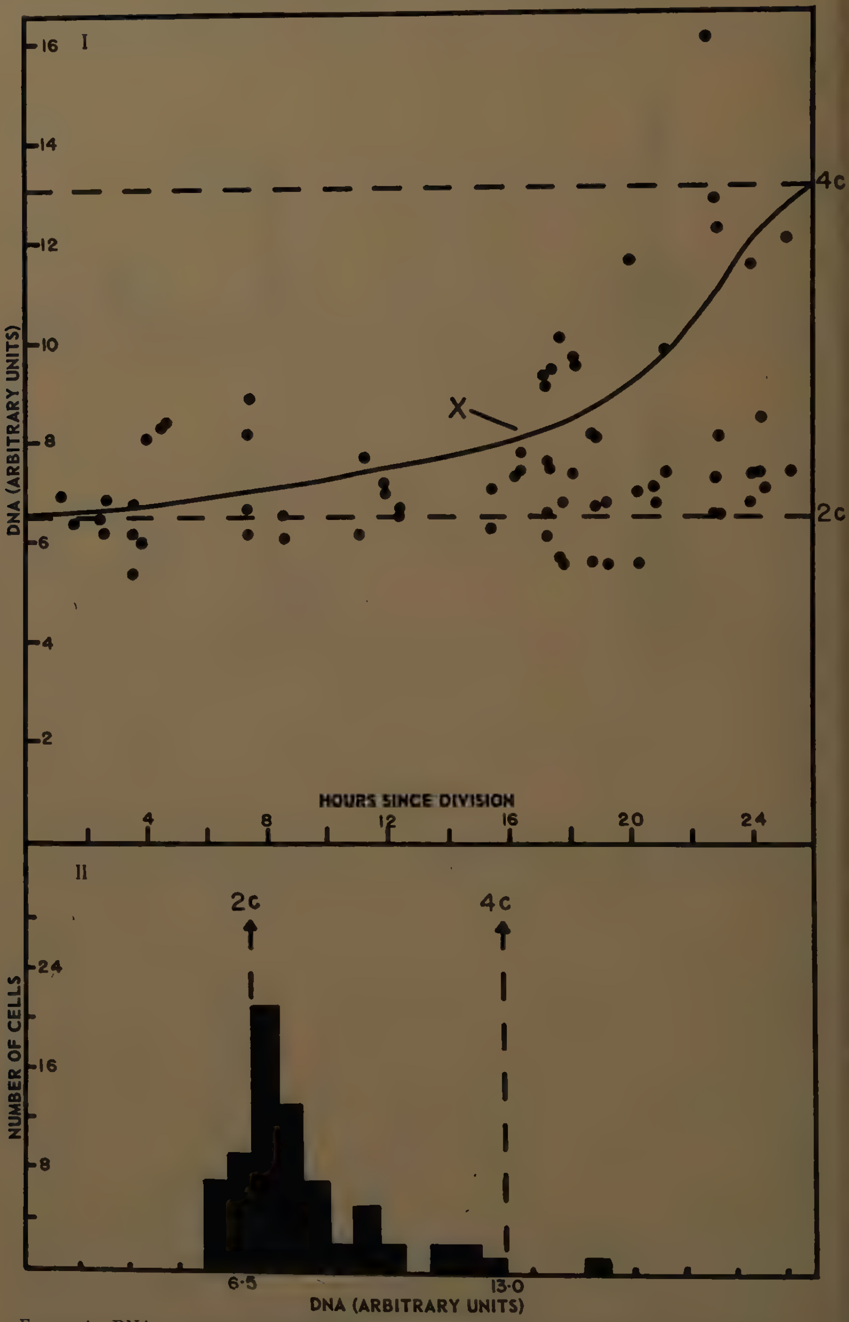

FIGURE 4. DNA amounts and synthesis in normal embryonic mouse cells grown in tissue culture. (I) Plot of DNA amount against time (in hours) elapsed since division. Each point represents one cell. Curve at X is the average DNA synthesis curve derived from (II) the frequency histogram of this population.

nuclei unsuitable for counting chromosomes. This advantage, together with the possibility of measuring DNA at mitotic stages other than metaphase, indicates that the method could be complementary to the usual chromosome counting procedures.

(3) DNA Synthesis in Embryonic Mouse Cells grown in Tissue Culture

Mouse embryo heart and lung tissues were grown by a modification of the hanging-drop technique due to Fell, which has been described elsewhere.[14] The cultures were studied by cinematography, together with measurements of Feulgen stain in which an earlier method of measurement was used (see Walker and Yates[14]).

FIGURE 4 illustrates (I) a plot of DNA values (at the time of fixation) of the cells that divided during the period of filming against the time (in hours) that had elapsed since each cell divided. Measurements of cells about 15 to 20 hours following mitosis show a range of values between the 2c and 4c amounts, and were therefore made during the period of DNA doubling. Other cells still contain the 2c amount long after their last division, thus indicating that these cells are probably going to divide after a somewhat longer interphase with their period of doubling beginning later. The frequency histogram in FIGURE 4 shows (II) a large peak at the 2c value, correlated with the lag period prior to the beginning of synthesis, and a smaller peak associated with a similar period before mitosis. The curve at X (FIGURE 4) is the average curve for the synthesis of DNA that has been calculated from the histogram (II) in FIGURE 4. It shows good agreement with most of those measured cells which have intermediate values of DNA.

While, in these particular cells, synthesis occurs late in interphase, the time required for DNA doubling is approximately the same as that in cells of chick grown in tissue culture that have a much shorter intermitotic period (~12 hours). As already mentioned, these measurements were made with an earlier and less accurate method, but the interphase synthesis is illustrated, we believe, rather strikingly by FIGURE 5, in which the interphase values obtained by the scanning procedure lie almost entirely between 2c and 4c.

The quantitative behavior of DNA in normal dividing cells of mouse tissue cultures is thus reasonably clear. We have examined also adult and embryonic tissues of the mouse in smear preparations and found the characteristic distribution of amounts of DNA in agreement with our data on tissue culture material and the data of Swift[13] on normal tissues of young and adult mice.

(4) Synthesis and Amounts of DNA in the Ehrlich and Krebs Ascites Tumors of the Mouse

Samples of these tumors were obtained from Professor P. C. Koller and Doctor Alma Howard, and stocks are now maintained in white mice in our laboratory. Smear preparations on coverslips of fresh ascites were fixed by methanol freezing-substitution and stained by the Feulgen method.

FIGURE 6 illustrates (II) a distribution of interphase DNA values from the Krebs ascites tumor four days after transplantation. At least, during the

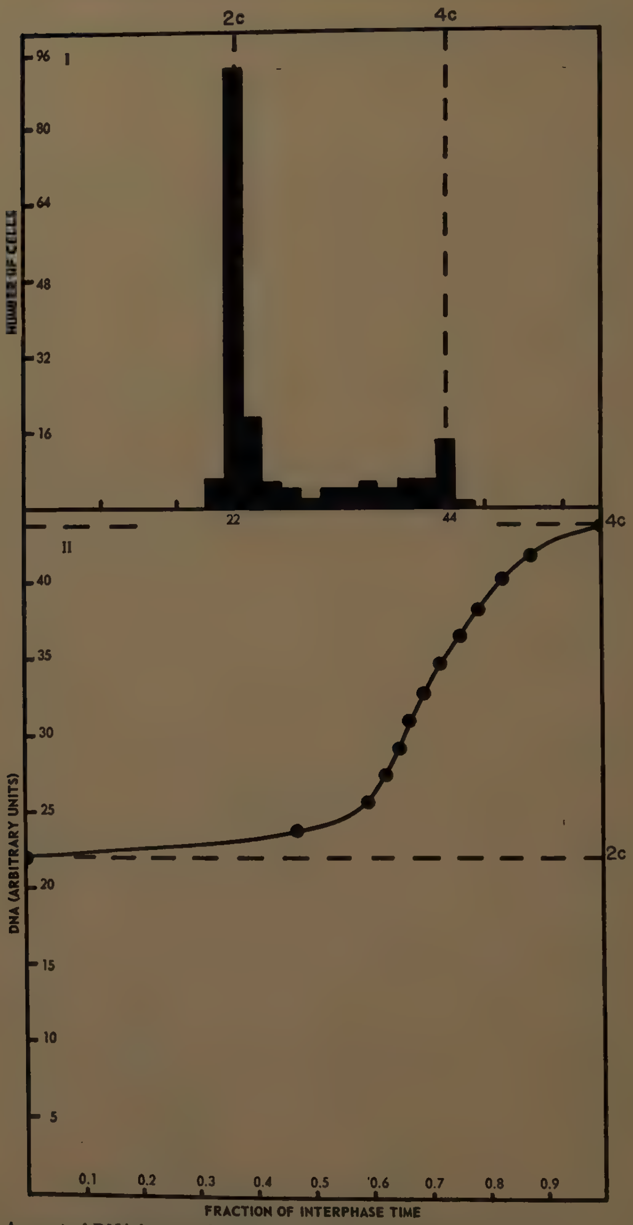

Figure 5. Amount of DNA in normal embryonic mouse heart cells grown in tissue culture. Photoelectric measurements. (I) Frequency histogram. (II) Calculated average synthesis curve.

beginning of the period of rapid growth, there are few aneuploid DNA values in the interphase sample. This is an important point that will be discussed later, but it also shows that we may be justified in calculating the average DNA synthesis curve for a sample of this population. It is even possible that the

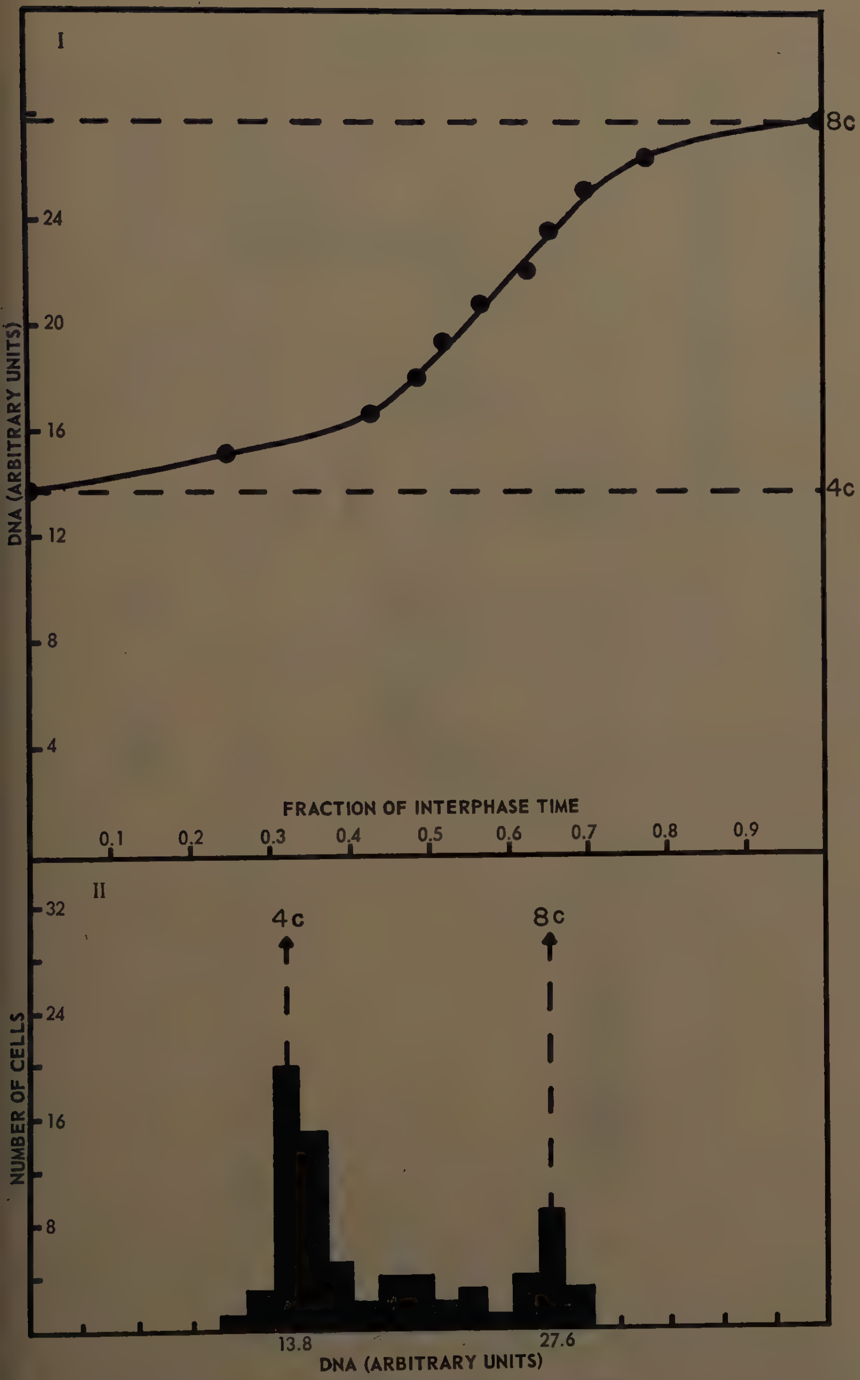

FIGURE 6. Interphase distribution of DNA values in the Krebs ascites tumor four days after transplantation. (I) Calculated average synthesis curve for DNA derived from histogram in II. (II) Frequency histogram of DNA values of the interphase population.

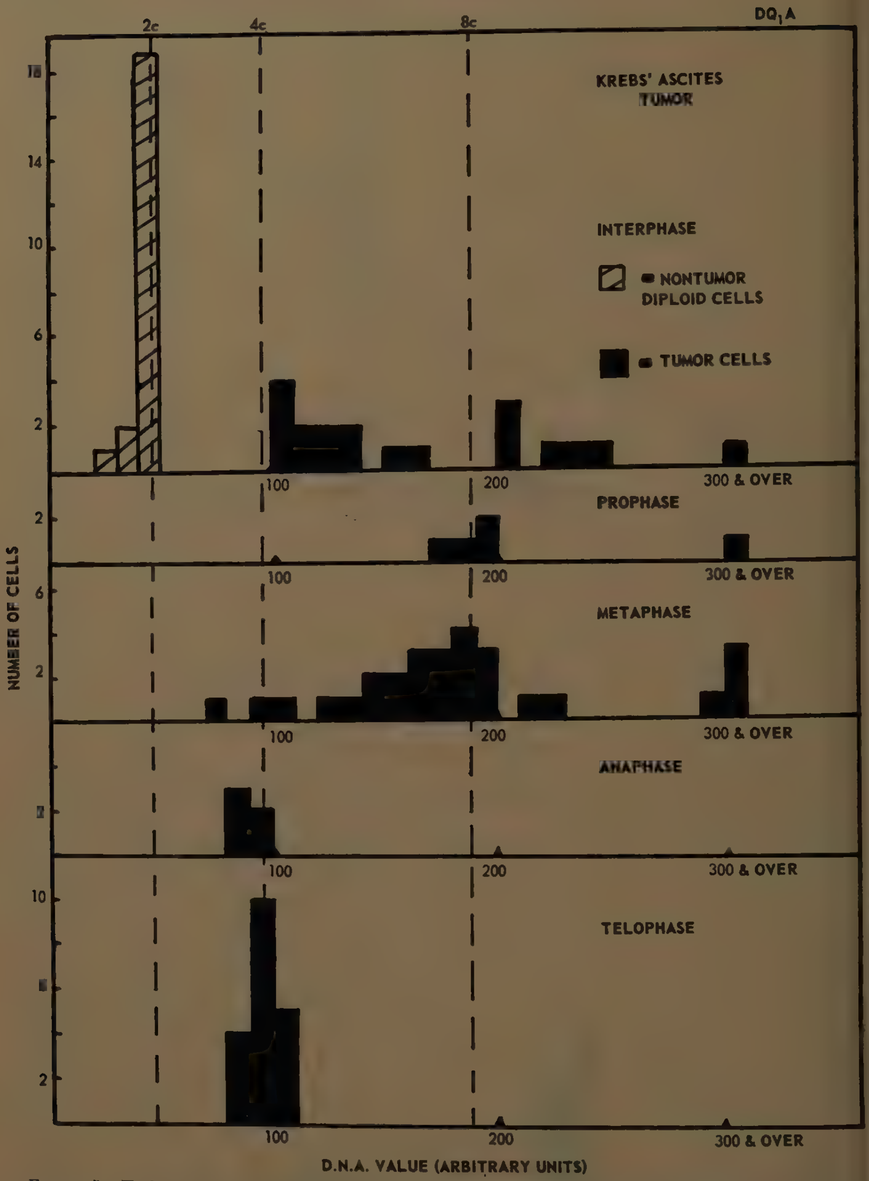

FIGURE 7. Krebs ascites tumor one day since transplantation. DNA measurements on stages in cell division. Black: tumor cells. Shaded: Nontumor inflammatory leucocyte cells as reference diploid DNA value.

accuracy of the calculated curve is greater in this material, owing to the uniformity of the cellular environment.

The calculated synthesis curve from the interphase sample is plotted (I) in FIGURE 6. It is very similar to that found for normal mouse tissue culture

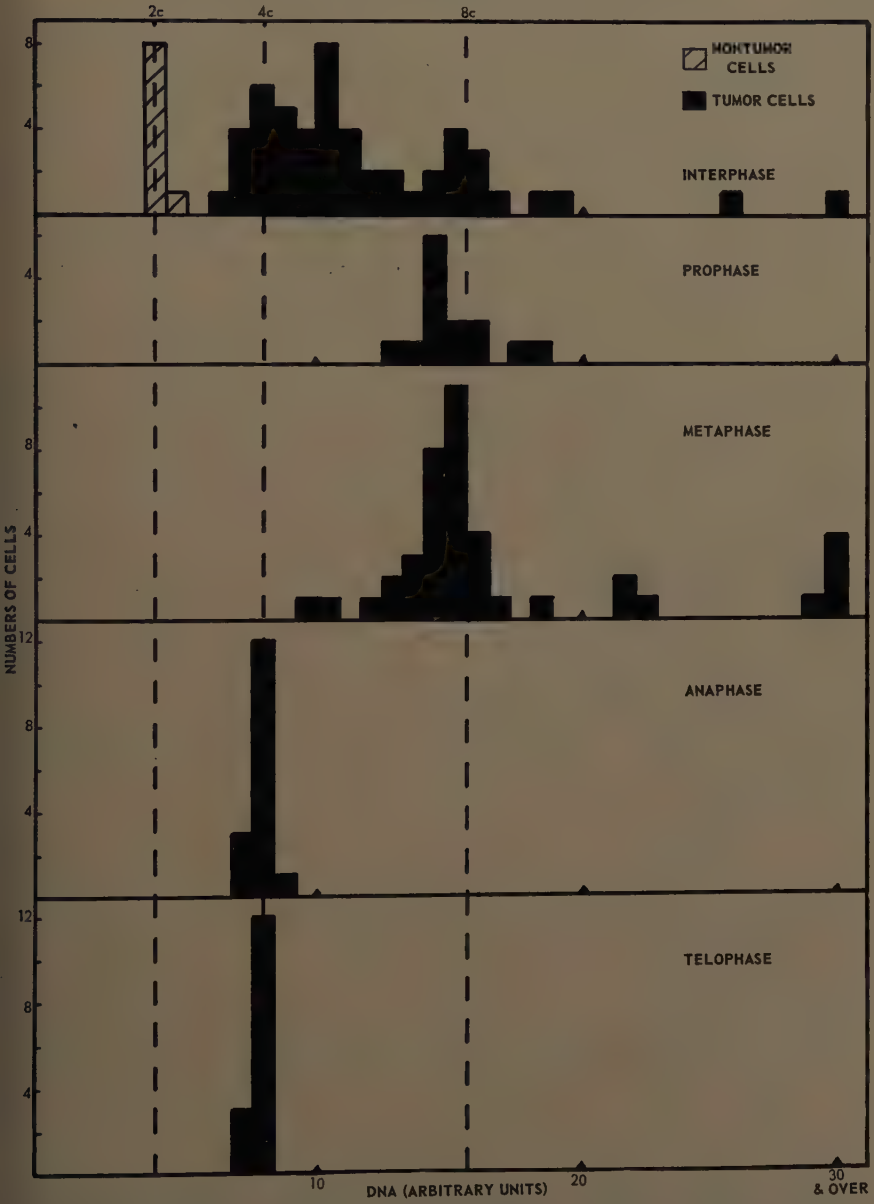

FIGURE 8. Ehrlich ascites tumor five days after transplantation. DNA measurement on stages in cell division. Black: tumor cells. Shaded: Nontumor inflammatory leucocyte cells as reference diploid DNA value.

cells (FIGURE 5), but does not agree with the findings of Klein, Klein, and Klein[32] on Ehrlich ascites cells.

Measurements of DNA in the Krebs and Ehrlich ascites tumors for all mitotic stages are illustrated in FIGURES 7 and 8 (taken from Richards[24]).

Certain deviations from the values for normal tissues (FIGURE 5) are evident. In both Krebs and Ehrlich ascites tumors, where the DNA content of inflammatory leucocyte nuclei is taken as the standard 2c value, there is a wide range of DNA content for metaphase cells (about the 8c value) in agreement with chromosome counts on the same tumors, but this is not reflected in a comparable range of values for telophase nuclei that show a very narrow variation about the 4c value. This result suggests that normal mitosis with successful cell cleavage is due mainly to cells that, at metaphase, contain amounts of DNA near the 8c quantity. This observation will now be considered in relation to the stem-cell concept of tumor growth.

(5) *Discussion*

The stem-cell concept. The stem-cell concept was inferred from observations by Hauschka and Levan[10] and Makino and Kanô[26] that in many ascites tumors, the metaphase populations are characterized by a mode of chromosome numbers maintained throughout many transplant generations. The concept has been supported by single-cell isolation experiments[27, 28] by chromosome analysis made by other authors[29, 30] and by our results presented here.

It has been considered probable[31] that the metaphase distribution of chromosome numbers reflects the degree of aneuploidy in the tumor cell population, but our interphase distributions of DNA values exhibit less aneuploid variation than might be expected on this basis. It may be, therefore, that aneuploid cells linger at metaphase, thus making a greater contribution to the sample of metaphases than to that of interphases. Since they do not appear to undergo cell cleavage, they must either reconstruct to form higher polyploid cells or binucleate or multinucleate cells which are not uncommon in these tumors, or they degenerate.

More interesting is the question of how the aneuploid cells arise. These cells may result from the occasional unequal division of an 8c metaphase, but morphologically normal telophases with abnormal DNA values have not been found. Incomplete or excessive DNA synthesis (or chromosome reduplication) during interphase is also a possible mechanism for the production of aneuploid cells. The few bipolar and multipolar divisions seen in these tumors, however, give rise to telophases that almost always have abnormal DNA values, while the sum of the DNA content of the telophases arising from a bipolar or multipolar division regularly approximates to 8c or, sometimes, 16c. Such abnormal telophases may, after an interphase period, re-enter mitosis to form aneuploid metaphases, and this seems to be a likely source for the aneuploid cells. It may be difficult, however, to decide whether the frequency of multipolar divisions is great enough to account for the number of aneuploid metaphases present, unless, as we have inferred, the abnormal metaphases linger at this stage in division. Metaphase blocking by colchicine should cause a greater accumulation of stem-cell metaphases than aneuploid metaphases. In our experiments, while there was a greater increase of stem-cell than of aneuploid metaphases in a colchicine-treated tumor sample, the results were unconvincing since blocking is not absolute and reconstruction follows. FIGURE 3 (BI and

BII) is interesting in this respect, since no metaphases much below 8c were encountered in this sample, but aneuploids between 8c and 16c were abundant. This phenomenon requires further analysis.

The observations we have presented lend support to the stem-cell concept, and suggest that aneuploid cells are a by-product of tumor growth, the usefulness of which may be small in an environment compatible with the genotype of the stem cells.

Quantitative cytochemical methods and the analysis of cell populations. We have shown that we are now able to distinguish with some assurance among chromosome number, synthesis, and inaccuracies of technique as factors causing variation in the amounts of DNA measured by the Feulgen method. The analytical method we have outlined can also be used, in principle, with other quantitative techniques, at present few, that are applicable to single cells.

It is possible to analyze a population heterogeneous with respect to certain cell components, and to understand more clearly the effect of the experimental modification of the cell processes involving these substances. In particular, we have seen that the spread of results so often obtained can be made, with suitable methods, to yield more information about the variable behavior of cell constituents than is implied by the common practice of publishing average values for such a sample. This practice, which we believe to be misleading, should be confined to homogeneous cell populations only.

It may be more justifiable to assume that cells of a population behave alike and that, for example, DNA synthesis occurs at a similar period during interphase in most of the cells of a population when a frequency histogram can be used to calculate the times and rates of synthesis. Further experiments will show whether these methods can produce fruitful results, and they may enable us to understand those processes which, by initiating and modifying DNA synthesis, are probably connected with the factors that cause cells to divide.

Acknowledgments

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Discussion of the Paper

Doctor M. J. Moses* (*Rockefeller Institute for Medical Research, New York, N. Y.*): There are two points raised by Doctor Walker's excellent paper that I think are worth mentioning:

The first point is methodological and concerns the cell "crushing" method used to flatten such heterogeneously absorbing structures as division figures in order thus to reduce extinction effectively. When the entire flattened structure is included in the field to be measured photometrically, the errors due to heterogenous distribution of absorbing materials that tend to lower the observed amount of absorber from the true one are only minimized, not avoided. This gain is at the cost of working at very low extinctions where the influence of other errors, particularly nonspecific light loss, and variations in source intensity and of phototube-amplifier response becomes appreciable. When the field is scanned by a small spot as in the work just presented, however, and the total absorption is integrated, the reduction of high extinctions to lower ones of the order of 0.3 to 0.6 (where the cytophotometric method becomes most sensitive) by judicious flattening of the object, with careful use, could be of considerable advantage.

The second point concerns the significance of variations in the Feulgen content of nuclei. I refer now to the experimental evidence for the stem-cell concept in which a wide spread of metaphase Feulgen values occurs that does not appear in telophase or early interphase nuclei. Observed variations in Feulgen content may be attributable to either experimental or biological factors, and it is often difficult in the absence of *a priori* evidence to decide which is responsible. Assuming that measuring procedures contribute little to the variation,* differences in Feulgen content may still be due to changes in the stoichiometry or absorption spectrum of the DNA-Feulgen complex as well as to such biological variation as DNA synthesis, loss of DNA, exceptions to DNA "constancy" or variations in chromosome number. Choice among these rests heavily on independent evidence such as chromosome counts, *etc.*

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In our experience, average values for DNA content agree well with hypotheses based on pre-existing evidence. For example, in a polyploid series of tomato where chromosome counts show plants to be n, $2n$, $3n$, and $4n$, the DNA (Feulgen) content is proportional to degree of ploidy.[3] The distribution of values within a population of cells, however, is sometimes variable, sometimes constant, depending on the tissue. In tomato leaf palisade layer nuclei, Feulgen distributions are homogeneous and unimodal, while, in the neighboring epidermal layer, wide spreads are observed and the possibility of systematic intermodes is strongly suggested. This would indicate a regular variation in Feulgen content of the latter nuclei that often yields values below the mean for the class representing the smallest chromosome complement. Similar spreads and regular intermodes have been observed in certain adult rat liver nuclei and, even when nuclei are measured by the two-wave-length method, the intermodes persist, although they are difficult to prove significant by statistical methods. In the absence of independent evidence, one cannot ascribe, with complete justification, a particular biological significance to these deviations. In this regard, Vincent[4] has reported that the Feulgen content of paired liver nuclei varies as much as 48 per cent and that the variation among cells is as great. Is this variation biological?

An analogy to the case in ascites where there is variation in Feulgen content of metaphases and prophases but not of telophases was encountered in measuring the Feulgen-DNA content of synchronized *Tradescantia* microspores by the two-wave-length method.[5] Distributions of early interphase values are homogeneous (synthesis does not occur until the end of interphase) and unimodal with small spread (S.E., ±3 to 4 per cent), but the mean values of the preprophases and prophases are consistently 10 to 15 per cent higher than the expected. As the interphase proceeds, the S.E. progressively increases to about ±10 per cent. The telophases resulting from the division, however, are equal to the preceding early interphases with a low S.E. Is this variation biological? Here, it cannot be ascribed to chromosome variation, and there is no other apparent reason for DNA content to vary here.

Two pieces of evidence make the data presented by Doctor Walker convincing: the linear relationship between chromosome number and Feulgen content, and the resemblance between chromosome and Feulgen distribution curves. However, a question is raised by the fact that, if the metaphase Feulgen variations are indeed all due to chromosomal anomalies and the aneuploid nuclei are doomed to stasis and will restitute to form approximately 8 C cells, then a fair number of these cells could be expected to appear as interphases in the 8

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* The methods described by Doctor Walker and the two-wave-length method minimize such errors.[1,2]

C class. While 8 C cells were reported, they cannot be distinguished from cells that were synthesizing or had completed synthesis. In the ultraviolet data of Leuchtenberger, Klein, and Klein,[6] as well as in their biochemical results, Erlich ascites interphase cells have the 4 C amount of DNA with few if any 8 C cells. It is possible, of course, that the latter data represent a more mature or otherwise different population, but it is necessary in any case to suppose, without strong evidence to support it, the demise of the restituted cells.

It is possible that the variations at metaphase and prophase in ascites and in the prophases of *Tradescantia* represent differences in the Feulgen reaction (which may, however, in turn have an interesting biological basis). This painful but necessary consideration is not entirely ruled out by the very careful and admirable work presented by Doctor Walker.

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FILM DEMONSTRATION OF *IN VITRO* GROWTH OF THE YOSHIDA ASCITES SARCOMA

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The Yoshida tumor was originally found in 1943 in one of 10 rats that Doctor T. Yoshida had fed for a three-month period with amidoazotoluol. The rats were also painted afterwards for four months with a solution of potassium arsenite in alcohol (Fowler's solution). This tumor was first seen in the scrotum. Autopsy showed a milky ascites. The tumor has been transplanted from animal to animal as an ascites tumor since its origin. Yoshida assumed that it is a sarcoma of the reticuloendothelial system.[6]

In the *in vitro* work reported here, the ascites tumor was inoculated subcutaneously in young rats. The solid tumor produced in about 10 days was cut into small fragments about one cubic millimeter in size and explanted into Carrel flasks. The medium contained 40 per cent chicken plasma and 60 per cent of a culture mixture of four parts of balanced salt solution (Pannett-Compton modified by Lettré), two parts chicken embryo extract (50 per cent), and four parts of human placental cord serum. The same culture mixture was used as the supernatent fluid. It was changed three times a week.

At first, a tremendous migration of macrophages occurred. Large spindle cells followed. The macrophages disappeared almost completely after the second transfer. During the first three days following the transfers, there was produced an apparently pure fibroblast culture. Shortly thereafter, a wave of very rapidly growing ameboid cells migrated from the central area and these appeared to wander on top of the fibroblasts. Usually, they showed no desire to leave their fibroblastic carpet. Cells that went beyond the edges of the colony usually returned to the fibroblasts. Others did not return. When such cells remain isolated from the fibroblasts, they will not grow independently.

Between the third and the tenth days, the multiplication of the ameboid tumor cells reaches its highest point. After this, the basic fibroblast colony is covered by a dense layer of these ameboid tumor cells, which soon extends beyond the border of the fibroblastic colony. Most of the cells that lose their contact with the fibroblasts soon perish.

In a study of the tumorous nature of these cells, animal inoculations were carried out. When free, cells were taken out of the culture flasks by pipette and injected intraperitoneally into 10-day-old rats. Even in small groups of five to six cells, they induced in 90 per cent of the animals a very rapidly growing ascites tumor with death of the animals after 8 to 10 days. The tumorous animals showed 2 to 3 cc. of an hemorrhagic ascites with heavy infiltration of the peritoneal cavity and often with icterus.

In order to establish proof that the ameboid cells were the malignant ones, we tried to grow isolated groups of tumor cells without fibroblasts. These groups of round malignant cells ceased to divide within 8 to 10 days after explantation. At this time, when the tumorous degenerating cells were still very abundant, we added a normal rat fibroblast colony embedded in clot to the roller tubes on the opposite side of the tumor cell colony. This produced a dramatic result. Twenty-four hours later, the recovery of the tumor cells was almost complete, yet there was still no direct visible contact with the fibroblast culture.

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We tried to substitute for the living fibroblast colony the cell-free centrifuged fluid from a rat normal fibroblast culture that had been in contact with the fibroblasts in a roller tube for 24 hours. The tumor cells died in this medium as rapidly as in the regular medium. Other attempts were made to feed them with varying concentrations of a cell-free extract prepared from muscle of newborn rats. These mesenchymal tissue extracts also failed to satisfy the tumor cell growth requirements.

Our preliminary observations demonstrate that the Yoshida tumor cells grow luxuriously in a kind of symbiosis with normal rat fibroblasts. These observations suggest that the Yoshida ascites sarcoma cells, at least *in vitro*, are not able to use the medium directly. These cells seem to depend on some product of the metabolism of normal rat fibroblasts. Additional experiments are contemplated to determine which factors are necessary to support the growth of these dependent tumor cells.

In 1954, Doctor Yoshida visited our laboratory in Heidelberg and suggested that we perform single tumor cell inoculations onto normal rat fibroblast cultures. At first, we inoculated single cells into some normal rat fibroblast cultures grown in Carrel flasks. We used essentially the method described by Yoshida. After 12 hours, an examination of the fibroblast cultures showed that in each case the inoculated single tumor cell had disappeared from view between the fibroblasts. Even during the next three to four days, no tumor cell could be recognized. At the end of the fourth day, however, many tumor cells were found in the area of inoculation of the single cell. At two weeks, the whole culture was overcrowded by tumor cells.

To improve our observations, the same experiments were repeated with slide cultures. In 15 single tumor cell inoculations onto fibroblast cultures grown on slides, we obtained 10 successful takes. Five other single tumor cell transfers missed the fibroblast colony completely and failed to grow.

The concept that some supporting cell different from the tumor cell is necessary for a good growth of certain tumor cells in culture has already been mentioned by Bichel[1] in connection with his work on mouse leucosis and by de Bruyn *et al.*[2] with mouse lymphosarcoma. In Bichel's experiments, however, the added normal tissue was used up at the expense of the tumor cell growth and therefore no symbiosis appeared to occur. In the experiments of de Bruyn *et al.* and in our own experiments, we are apparently dealing with a symbiotic cell relationship. In more recent work of de Bruyn *et al.*[3] another interesting phenomenon occured. A stable culture of malignant lymphoblasts growing with fibroblasts in symbiosis underwent a profound change with an eventual loss of the characteristic supporting fibroblasts, leaving only the "altered" lymphoblasts in culture. These tumorous round cells grew well without fibroblasts and, when inoculated into mice, they did not produce a tumor. The nature of these profound changes in this tumor cell population has not been revealed.

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CONTRIBUTIONS OF THE ASCITES HEPATOMA TO THE CONCEPT OF MALIGNANCY OF CANCER*

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The ascites hepatoma of the present study is prepared by conversion of the amino-azo-dye-induced hepatoma of the rat into the ascitic form, and is similar in all aspects to other types of ascites tumors of various derivations. Its chief traits, however, may be found in the following two points: (1) that it is reproducible; and (2) that the tumor cells evidently maintain epithelial characteristics while suspended in the ascitic fluid.

The Ehrlich ascites tumor, first of the ascites tumors, was prepared by ascitic conversion of solid Ehrlich carcinoma.[7] The Kleins have been engaged in attempts to convert various kinds of tumors into the ascitic form,[18-23] and Doctors G. and E. Klein have contributed a paper on this subject to this monograph. Almost all ascites tumors available at present are those converted from solid tumors at hand, either of established transplantable strains or of spontaneous ones, and they are not reproducible. The so-called Yoshida sarcoma of the rat, established in 1943, is an example of a primary ascites tumor, and attempts at its duplication have never been successful.[29, 30, 33]

A brief history of the production of the ascites hepatoma, which followed our attempts to reproduce the so-called Yoshida sarcoma, is as follows: as the Yoshida ascites sarcoma originated in one of the rats subjected to an experiment concerned with the production of hepatoma, we carried out careful histopathological examinations of the hepatoma tissue and assumed that the hepatoma tissue included, besides the main neoplastic cells of hepatic cell origin, other elements of Kupffer-cell derivation. Thus, the hepatoma presented in our interpretation is a kind of tumor composed of neoplastic cells of two different derivations, an analogy being found in the chorionepithelioma.[32] Then this was followed by attempts to isolate, from the hepatoma tissue, the Kupffer cell-derived neoplastic cells by means of ascitic conversion of the tumor, in the hope that eventual success would yield an ascites sarcoma of reticuloendothelial-cell origin, which might be the same in nature as the Yoshida sarcoma. This was why we described the Yoshida sarcoma in a previous paper as a "reticulum-cell sarcoma?"[33]

The experiment of transforming the hepatoma into the ascitic form was started in this way, and the resultant ascites hepatoma was at first subjected mainly to morphological investigations. The flat, elongated cells found on the surface of "hepatoma islands," cell aggregates suspended in the ascitic fluid, were regarded at first, and naturally by us, as those of Kupffer-cell derivation, which also had a neoplastic character (FIGURES 1 and 4). The so-called

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* This investigation has been carried out in collaboration with Doctors Hidehiko Isaka, Kyuya Nakamura, and Shigeyoshi Odashima (Medical Institute of Sasaki Foundation, Tokyo) and Doctors Hiroshi Satoh and Hiroshi Imamura (Iatrochemical Institute of the Pharmacological Research Foundation, Tokyo). The work of Doctor Haruo Sato and of Doctor Tomohiko Aruji, former associates in the Tohoku University, Sendai, Japan, provided the basis for various points of discussion.

TABLE 1
THE ASCITES HEPATOMA

Strain	Established	Generation	Number of animals transpl.				Rate	Survival time in days*
			Total	Valid	+	—		
130	June 1951	1–149	519	487	469	18	96.3	14
7974	July 1952	1–122	319	300	289	11	96.3	13
601	Aug. 1952	1–59	205	180	139	41	77.2	28
602	Sept. 1952	1–50	198	170	134	36	78.8	27

* Median value.
February 1955, Sasaki Institute.

‘pairs’’ were considered by us to be a symbiotic combination of two cancer cells of both hepatic and Kupffer-cell derivations (FIGURE 2). However, the ater success of the transplantation of the ascites hepatoma with a single cell compelled us to correct the above interpretation of the cellular constitution of the ascites hepatoma.[34, 14] The inoculated single cells could be nothing more han hepatic tumor cells, presenting, in their further growth, cell associations characteristic of epithelial cells (FIGURE 3). In such one-cell-derived tumor ascites there also appeared typical pairs attended by endothelial-like, crescent-shaped cells. On the surface of larger islands, there were also found flattened endothelial-like cells that were now definitely a mere morphological adaptation of superficially located epithelial tumor cells to their surroundings (FIGURES 1 and 4).

We thus failed in the attempt to reproduce the so-called Yoshida sarcoma, but the ascites hepatoma has proved itself to be useful in cancer studies, providing a tool for comparative studies of ascites tumors of common normal ancestry.

Conversion of Solid Hepatomas into the Ascitic Form

In June 1951, the first transplant strain (AH 137) of the ascites hepatoma was successfully established in our laboratory and was followed by five other strains* in about one year.[32, 26, 13, 17] Four of them have been kept, up to now, or more than 50 to 150 transfer generations (TABLE 1). Two of the four strains (AH 130 and 7974) were derived from different azo-dye hepatomas induced n two different animals, while the remaining two (AH 601 and 602) are substrains derived from a hepatoma originating in a third animal.

The procedure of ascitic conversion of the solid hepatoma is rather simple, although the yield so far hardly exceeded 10 per cent of all the trials (TABLE 2). The method of hepatoma induction in the rat is, in principle, the same as that described in 1935.[25] The substances we employed were either o-amino-azo-oluene (AAT) or p-dimethylamino-azo-benzene (DAB). AH 130 and 7974 were derived from DAB-hepatomas, and AH 601 and 602 from an AAT-hepatoma.

In order to produce hepatoma in the rat, it is our habit to begin azo-dye

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* AH 108, AH 130, AH 7974, AH 601, and AH 602.

TABLE 2

ASCITIC CONVERSION OF THE AZO-DYE-INDUCED HEPATOMA OF THE RAT

Dye	Animals	Nodules	Ascites	%
D.A.B.	29	46	4	8.7
A.A.T.	15	15	2	13.3
Total	44	61	6	9.8

feeding in relatively younger animals of 80 to 100 g. body weight. The animals are fed, in addition to rice mixed with azo dyes, raw vegetables, greens and occasionally dried fish. There are a number of detailed investigations on the relation between azo-dye hepatoma development and nutrition[1, 27] that are no doubt of significance in relation to the mechanism of carcinogenesis. For the mere purpose of hepatoma production, however, we do not seriously consider the nutrition of the animal with respect to the qualitative and quantitative aspects of the foods provided in the diet. Instead, foods are given *ad libitum* and then adjusted in kind and amount according to the habits of the rat under observation. Special attention, however, is paid to whether the animals are taking the expected amount of the azo compound. The azo dye mixed with rice is placed in a carefully prepared food box, where it cannot be easily scraped out, and the remains are regularly measured. When it is indicated that the amount of remaining rice mixture is too much, additional foods are regulated accordingly. On the other hand, the azo-dye feeding can be interrupted for several days in order to arrest an eventual deterioration of the general condition of the animals.

As a substratum food[4] with which to mix the oil solution of the azo compound, rice is our choice (it is important to crush the grains so that their adsorption of the oil solution is favorable), but barley (treated as above), crushed dry noodles, *etc.*, are useful as well.[4] The animals we use are noninbred or heterozygous albino rats. We usually start the experiment, as described above with 80 to 100 g. animals, although older animals weighing more than 200 g do not present essential difficulties. Animals younger than those weighing 50 g. cause much loss, in our experience, by failing to survive the beginning of the experiment.

The azo-dye feeding for four to five months is sufficient for the production of hepatoma.[25] Then the animals of 80 to 100 g. usually reach a weight of about 200 g. and, although signs of tumor development in some animals are not yet manifest, the feeding of the azo compound is best stopped here. In one to two months, during which the animals are left under ordinary living conditions, the tumor grows gradually. One will obtain, in this way, a well developed hepatoma as compared with the case of continuous feeding.

Animals bearing well-developed azo-dye hepatomas are sacrificed, and the tumor tissue is minced and inoculated into the peritoneal cavity of normal rats. In the case of multiple development of tumors, transplantation can be tried from each of the tumors separately. It may be noted that, in cases of fully developed hepatoma, animals often present spontaneous ascites that contains various amounts (sometimes a large number) of hepatoma cells (islands). The latter are primary ascites hepatomas. We succeeded once in establishing a transfer strain with such material (AH 130).

Results of transplantation with minced material from the same hepatoma tissue are not always the same in every animal inoculated. In one animal, successful ascitic conversion can result immediately, while in another, only tumor nodules develop on the peritoneal surface without showing significant accumulation of ascitic fluid. In the latter case, the same procedure is repeated using the nodules. AH 601 and AH 602 represent an example of these differences. Minced material of a solid hepatoma was inoculated, in this case, into eight animals, in one of which the hepatoma ascites developed immediately. From this material, a strain of ascites hepatoma (AH 601) was established. In another, only tumor nodules developed, which were converted into the ascitic form in the following generation, *i.e.*, in the second transfer generation (AH 602).[13] Another example of such "indirect" conversion was strain AH 137.[26] In this case, the conversion first succeeded in the fourth generation. The serial transplantation of this strain was abandoned after 51 transfer generations.

The results so far obtained in our attempts at transforming solid hepatomas into the ascitic form are summarized in TABLE 3. Among 61 histologically malignant hepatomas tried, 38 failed to "take" upon "intraperitoneal" transplantation. Of 23 "takes," 3 were successful in immediate conversion into the ascitic form. In the remaining 20 cases, further attempts at ascitic conversion were continued with tumor nodules that developed on the peritoneal surface and, in 2, the conversion was successful in the second (AH 602) and fourth (AH 137) generations respectively. In the remaining 18 cases, the same experimental procedure was continued until the seventh generation at most, but without success in any case. It was noted during the experiment, however, that, in several cases among the 18, there accumulated in the peritoneal cavity a considerable amount of ascitic fluid, but the ascitic fluid in these cases did not present, at death of the tumor animals, a pure culture state of tumor cells similar to the typical examples shown in FIGURES 5 to 8, and the animals died because of an increase in solid tumors rather than an increase in tumor ascites. We discarded these cases from those that showed successful ascitic conversion. Because the follow-up of these discarded cases was not sufficient, not exceeding seven transfer generations at most, it may not be proper to refer here to the problem of the "gradual" conversion described by E. Klein.[20, 23] In fact, we were not aware of this kind of biological problem, especially in the first half of the present investigation, until just recently, when

TABLE 3
TRANSFORMATION OF SOLID INTO ASCITES HEPATOMA

No. of Solid hepatoma tried	No. of "Takes"		No. of "nontakes"
	Solid	Ascites (immediately)	
61	20*	3	38

* Two among these were converted "indirectly" into the ascitic form.

TABLE 4

RESULT OF INTRAPERITONEAL TRANSPLANTATION WITH SPONTANEOUS ASCITES OF ANIMALS BEARING PRIMARY AZO-DYE HEPATOMA IN THE LIVER

Animal No.	Content of "islands"	Animals transplanted	No. of "Takes"	
			Ascites	Solid
130	+++	3	3	0
108	++	3	3	0
101	++	2	0	0
55	++	2	0	0
8560	+	4	0	1
8130	+	1	0	0
104	+	7	0	0
129	+	2	0	0
137	+	2	0	0
138	+	2	0	0
54	+	4	0	0
41	+	2	0	0
34	+	2	0	0
60	+	3	0	0
59	+	2	0	0
49	+	3	0	0
Total........		44	6	1

the informative work of the Kleins first appeared. It had been our naive impression that in the two cases (AH 602 and AH 137) of the "indirect" conversion described, the tumors had converted rather abruptly into the ascitic form after passing through generations of solid tumor formation.

As described above, we have noted a case of a primary ascites hepatoma but this was not the sole case of spontaneous ascites in animals bearing a hepatoma in the liver. So far, 23 such cases have been noted, but the content of tumor cells in the ascitic fluid, roughly estimated by the number of cell aggregates (islands) present, differed case by case within a fairly wide range. The intraperitoneal inoculation with this kind of ascitic fluid was tried in 16 out of 23 cases, in 3 of which it resulted in "takes" (TABLE 4), but the manner of the takes was not similar in all 3 cases. Two of the 3 rendered typical ascites hepatomas in all animals transplanted, and 2 strains were established from each of them, that is, AH 130 and AH 108. In the case of AH 108, another strain of ascites hepatoma was established by immediate conversion of the solid hepatoma from the same primary animal. Thus, this animal rendered two strains, one from the spontaneous ascites and the other by immediate conversion of the solid tumor.[26] In AH 108, serial transplantation had been continued until the 83rd generation, when it was abandoned, while AH 130 has been kept until now. In the remaining one (No. 8560), it was interesting to note that it "took" in the solid form, despite the fluid form of the inoculum. It is regrettable that further attempts at conversion with this solid tumor are lacking.

To summarize the results (TABLE 3): among 23 solid, primary hepatomas that proved themselves to be (intraperitoneally) transplantable, 3 were immediately, and 2 were "indirectly" converted into the ascitic form and, from each of them, a strain of transplantable ascites hepatoma has been established. In addition, hepatoma animals were noted that presented a spontaneous, primary hepatoma ascites, from which two strains of ascites hepatomas have also been established. The ascitic conversion of hepatoma was, therefore, successful in about 21 per cent of the cases when only transplantable hepatomas are taken into account.

Animals inoculated with the hepatoma-ascites present, beside accumulation of the ascitic fluid, ample solid tumors on the peritoneal surface, which invade into the surrounding tissues (FIGURE 11) and are identified easily as hepatoma in histological sections. Distant metastases of the tumor can occur but rarely, perhaps owing to the relatively short duration of life of the tumor animals.

At first, we were so hasty as to think that every ascites hepatoma might be the same by nature, and out of six established transfer strains, two were abandoned along the way. After that, we decided to continue the experiment in order to have more strains established. At present, 2 strains are being established, running into 6 and 10 transplant generations, respectively.

The difference between the solid and the ascites tumors is probably due only to the gross appearance of the tumor, which presents apparently different features in both forms of tumor growth. Under the microscope, when tumor cells alone are taken into consideration, tumor cells of the solid tumor are in direct contact with tissue fluid, which is essentially similar, physiologically, to the peritoneal fluid. Thus far, in our experience, the Yoshida ascites sarcoma can grow at every site in the body where cells are inoculated. In the solid form, the tumor cells are stuffed into meshes of the network of the stroma which (in its extreme form) can be a fine net of fibres. However, within the mesh of the stromal net, the cells, regardless of whether they might be in an isolated free-cell condition, or in cell complexes, as in the case of carcinomas, are exposed to the environment of the fluid. Thus, with regard to the tumor cells themselves, the environmental condition of the ascites tumor, *i.e.* the peritoneal cavity, is considered to be merely an enlargement of narrow spaces of other tissues in the body, which are usually represented by the subcutaneous tissue in the case of tumor inoculation. Our investigations on the ascites tumor, the free-cell tumor of Goldie,[5] or the "fluid tumor," as we called it previously,[30] were started about 1942[29] under the foregoing assumptions, which were based on analytical discussions of histological structures of the malignant growth.[30] The main purpose of the investigation was to prepare, with the aid of the fluid tumor, a tool of cancer research that possibly could serve in exact and analytical studies of cancer and in morphological and chemical, as well as biological, studies of cancer cells. This would include screening work for cancer chemotherapy, which needs accurate and dynamic evaluations of cytological effects of chemical compounds under test.[30, 31, 33]

The ascites sarcoma of the rat, the so-called Yoshida sarcoma, seemed to support the foregoing interpretation in various aspects, especially in that a single cell of the tumor can give rise to both solid and ascites tumors, merely according to the site of its inoculation.[15, 9, 10, 11] A subcutaneous solid tumor derived from a single cell originating in the ascitic fluid is provided with a fine stromal reaction and encapsulated by a connective tissue reaction of the host. Within this grossly solid tumor, however, the tumor cells grow, soaked in the tissue fluid, in the same way that they grow in the ascitic form of the tumor, except that an exceedingly large amount of the intercellular fluid medium of the latter appears in relation to the cellular elements. Tumor cells from this solid tumor, inoculated into the peritoneal cavity, give rise immediately to an ascites tumor.

The ascites hepatoma seems to have presented another illustration in support of the understanding of the gross forms of neoplasms. Since the hepatoma does not have any usual firm stromal tissue, but is provided only with the "blood space" as the stroma,[28] tissues of "solid" primary hepatomas may be accepted as those composed of "cancer nests" covered by endothelial layers and soaked in the blood fluid. The condition of the ascites hepatomas is a mere modification of such a "histological" structure, where "naked" nests, without the barrier of endothelial layers, are directly surrounded by the peritoneal fluid instead of the blood. The cancer nests of the ascitic fluid that we call islands are prevented by a large amount of fluid medium from forming into a compact composition like the solid tumor, and they can show a gradual transition to single cells through intermediary stages of smaller and smaller islands such as three- or two-celled ones, as will be discussed later (FIGURES 9 and 10).

However, stress should be laid on the fact that every histologically malignant tumor is not always convertible into the ascitic form. As described above, only 21 per cent of malignant (transplantable) solid hepatomas could be converted into the fluid form. In the remaining cases, even when ascitic fluid accumulated in the peritoneal cavity, only small amounts of cells were suspended there, and this fluid alone could not be used for further transplantation. Attempts to find essential differences histologically, between convertible and inconvertible hepatomas have been made but, so far, definite results are not available. Here may be found, therefore, an important problem beyond mere morphological considerations, to be pursued by the aid of biological investigations.

Morphology of the Ascites Hepotoma

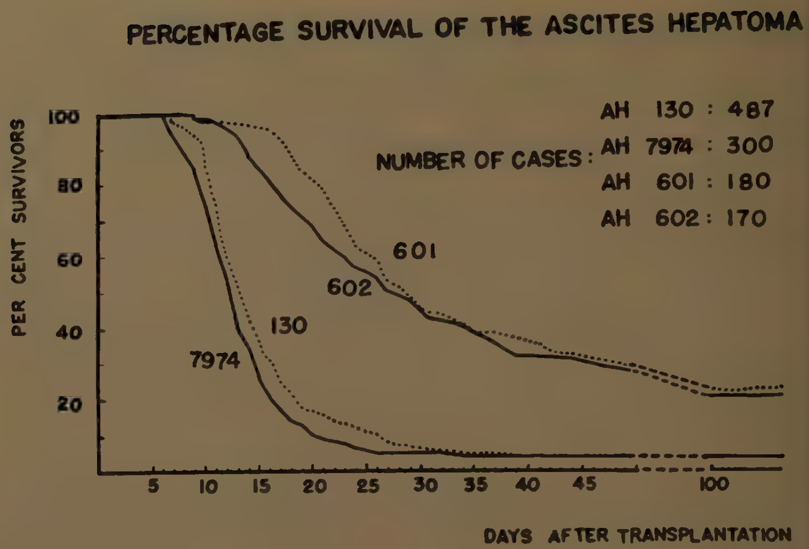
The ascites hepatoma is not a free-cell tumor in the strict sense of the word. The tumor cells, being epithelial in nature, make, as a rule, cell associations suspended in the ascitic fluid, but individually isolated cells are also present. The amount of these isolated cells differs by strain, but not by tumor animals in the same strain (*v.i.*). The formation of cell associates presents its typical feature in the beginning of tumor growth after transplantation. Several cells are arranged in a beadlike series, or gather in clumps that, growing to various sizes, may then be called "islands" (FIGURES 1, 3, and 4). Surface cells of the islands are sometimes flat, elongated, and hard to differentiate from endothelial elements (FIGURES 1 and 4). Actually, however, they represent an adaptation of epithelial cells to their location, contrary to our former interpretation, which held them to be Kupffer-cell derivatives (*cf.* discussion in the first section of he present paper). Islands, therefore, are cell aggregates composed exclusively of epithelial cells. In the "pairs," composed of two tumor cells, one ccasionally presents a crescent shape, nearly enclosing the partner (FIGURE 2), out actually it is also a transformation of an epithelial cell. The bonds that old both cells of the pair together vary widely in their fastness (FIGURES 9 nd 10).

It is difficult to stain the islands with Giemsa solution or the like. For the emonstration of the hepatoma ascites, acetogentian violet or acetocarmine taining is, according to our experience, the best reagent. A droplet of tumor scites is mixed on a glass slide with an approximately equal amount of the ye solution and covered lightly with a cover slip that can be sealed with alsam paraffin. The tumor cells are fixed with acetic acid and stained at the ame time with the dye. Islands of various sizes, as well as isolated cells, are tained beautifully (FIGURES 5 to 8). The preparation is useful as such for hromosome study (FIGURES 12 to 15).

The staining solution is prepared as follows: 0.75 g. of gentian violet is dissolved in 30 cc. of boiling glacial acetic acid, left until cold, and then added to 0 cc. of distilled water and filtered. Gentian violet, of course, can be replaced y other dyes, such as orcein, carmine, or the like. Since the preparation is ot stable, being useful for only about one week, we usually employ in our aboratory a modification of this method following the suggestion of Nakamura. The ascitic fluid is poured into a small glass tube usually containing two to three volumes of dye solution but which may be adjusted to any proportions esired. The tumor cells are deposited at the bottom of the tube, and a dropet from the deposit is ready for microscopic examination in the manner described above. The advantage of this method is that the cell deposit within the tube is useful for a long duration. Nakamura began the procedure about ne year ago, and a droplet tapped from the one-year-old deposit kept at room emperature offered a picture comparable to that of fresh material. Of course, he tube is portable, so that a fresh preparation may be had anywhere at any ime desired. For chromosome study it is of special use, since it enables repeated examinations of tumor cells that belong to one and the same ascitic luid.

Pictures of the hepatoma ascites differ considerably according to the strain, nd the appearance of each strain presents a characteristic picture. Among he present four strains, islands of strain AH 601 are very large. They are ompact cell aggregates and their surfaces look smooth (FIGURE 5). In strain H 602 (FIGURE 6), islands are also large, but they are more irregularly shaped nd the bonds of the cells seem to be loose. Strain AH 7974 (FIGURE 7) presents uniformly small islands and, between these islands, are pairs and small slands composed of three or four cells, as well as individually separated cells. These isolated cells are most abundant in strain AH 130 (FIGURE 8), and the slands are smaller and fewer than in the above, so that some parts of the picture ook like an ascites sarcoma.

The fact that the pictures of strains AH 601 and AH 602, which are two subines from the same hepatoma, are not identical, together with the fact that



(FEB. 1955 : SASAKI INSTITUTE)

CHART 1

both tumors have been converted into the ascitic form in different ways, one directly and the other indirectly (*v.s.*), may support the idea that the cell popu- lation of the "primary" hepatoma nodule was not a uniform one. This re- sembles, to some extent, the "mosaic concept" of Hauschka,[6] which is con- cerned mainly with established transfer strains of tumors.

Growth Pattern and Transplantation Rate

Strains AH 130 and AH 7974 have passed through 149 and 122 transplan generations respectively, and the average survival time of tumor animals is 14 and 13 days respectively (TABLE 1). They are tumors of relatively rapid growth, and individual differences in the survival time of tumor animals are not significant, as shown in the diagram of the percentage survival of tumor bearing animals (CHART 1). These two strains are closely similar, even though their primary tumors originated in two different animals. Strain AH 601 and AH 602, running into 59 and 50 transplant generations respectively, grow more slowly as compared with the above two strains. The average surviva times are 28 and 27 days, showing relatively wide individual variations (CHAR 1). The rates of transplantation of both strains, AH 130 and AH 7974, are equal, that is, 96.3 per cent, while the rates of strains AH 601 and AH 602 show a slight difference, 77.2 and 78.8 per cent respectively. In tumors of rapid growth, the transplantation rate is high, while it is relatively low in those of slower growth. That the differences between AH 601 and AH 602 in both respects look larger than those between AH 130 and AH 7974 may be due to a larger individual difference of tumor animals in more slowly growing and less

TABLE 5

RATE OF TRANSPLANTATION AND SURVIVAL DAYS IN THE FIRST AND THE LATEST 10 GENERATIONS OF SERIAL TRANSPLANTATION

Ascites Hepatoma: Strain 601

Generation	Rate of transplantation "Takes"/animals	Survival days Mean (min.-max.)
1st	4/6	28 (21–41)
2nd	2/2	31 (29–33)
3rd	5/6	30 (12–53)
4th	2/6	28 (24–32)
5th	2/4	24 (22–26)
6th	1/3	29
7th	3/3	26 (13–24)
8th	3/4	45 (23–88)
9th	3/3	37 (23–46)
10th	1/2	31
Avg.*, the first 10:	26/39 (66.7%)	31 (12–88)
he latest	2/2	28 (16–40)
2nd from the latest	2/2	35 (18–51)
3rd "	2/3	69 (48–89)
4th "	3/3	25 (23–26)
5th "	3/3	28 (27–29)
6th "	1/1	54
7th "	2/2	24 (23–24)
8th "	2/2	42 (41–42)
9th "	3/3	47 (44–55)
0th "	3/3	32 (28–43)
Avg., the latest 10:	23/24 (95.8%)	37 (16–89)

*As of March 1, 1955.

ransplantable tumor strains. Most remarkable, however, is the difference etween both pairs of tumor strains, as illustrated in CHART 1.

In TABLES 5 to 8, survival days and the transplantation rate of each strain 1 the first and in the latest 10 transfer generations are indicated. It is hard o realize from this that the growth tempo and the transplantation rate, with he advance of transfer generations, have increased gradually together, espeially when separate generations are examined one by one. These observaions may imply that these characteristics are fixed in each tumor strain from s onset.

Transplantation of the Ascites Hepatoma with a Single Cell

The experiment of single-cell transplantation was carried out employing train AH 130, which is the richest in isolated single cells of all four strains. he procedure, in principle, was the same as in the case of the Yoshida saroma,[15, 9, 10, 11] but so far we have had only 3 successes out of 98 valid cases ith the ascites hepatoma (TABLE 9). The rate of success in this case was ery low as compared with that of the Yoshida sarcoma, in which we could ttain 55 per cent success in single-cell transplantations.[10] In successful cases, umor growth was confirmed in about 15 days after inoculation. Animals in

TABLE 6

RATE OF TRANSPLANTATION AND SURVIVAL DAYS IN THE FIRST AND THE LATEST 10 GENERATIONS OF SERIAL TRANSPLANTATION

Ascites Hepatoma: Strain 602

Generation	Rate of transplantation "Takes"/animals	Survival days Mean (min.-max.)
1st	3/3	16 (14–20)
2nd	1/2	21
3rd	2/5	20 (16–23)
4th	3/4	23 (19–73)
5th	2/4	24 (23–25)
6th	4/4	27 (18–38)
7th	4/4	49 (27–108)
8th	2/3	26 (23–28)
9th	5/6	40 (9–120)
10th	6/6	13 (8–17)
Avg., the first 10:	32/41 (78.0%)	26 (8–120)
The latest	2/2	19 (14–21)
2nd from the latest	1/2	18
3rd “	2/2	21 (14–27)
4th “	3/3	54 (47–60)
5th “	3/3	19 (14–28)
6th “	3/3	38 (34–44)
7th “	3/3	23 (12–29)
8th “	3/3	36 (24–48)
9th “	2/3	44 (39–49)
10th “	2/2	37 (36–38)
Avg., the latest 10:	24/26 (92.3%)	32 (12–60)

As of March 1, 1955.

the three successful cases died of ascites tumor growth in 34, 40, and 46 days respectively after inoculation.[14]

From each of these three animals, three single-cell-derived, clonal substrains have been established: SH 1, 2, and 3, respectively. These substrains have been kept through more than 50 transfer generations. The rate of transplantation and survival days of tumor animals of the three substrains are similar to each other and identical with those of the mother strain, AH 130, as shown in TABLE 11 and CHART 2. Furthermore, it is completely impossible to distinguish the tumor picture of the mother strain from those of its three daughter clones, which are in turn identical with each other. The chromosome number of these four strains, as will be described in the following section, has also demonstrated a surprising uniformity.

Prior to the single-cell transplantation experiments, inoculation with various numbers of isolated cells was tried (TABLE 10). As shown in the table, increasing numbers of cells inoculated did not ensure tumor "takes."

The Chromosome Number

In each strain, a total of 200 metaphase plates were examined for their chromosome number, and the results were compared with each other. The examina-

TABLE 7

RATE OF TRANSPLANTATION AND SURVIVAL DAYS IN THE FIRST AND THE LATEST 10 GENERATIONS OF SERIAL TRANSPLANTATION

Ascites Hepatoma: Strain 7974

Generation	Rate of transplantation "Takes"/animals	Survival days Mean (min.-max.)
1st	5/5	16 (12–20)
2nd	4/4	15 (8–23)
3rd	2/2	14 (13–34)
4th	4/4	18 (10–28)
5th	1/1	12
6th	2/2	14 (10–17)
7th	2/2	16 (14–18)
8th	2/2	18 (18–18)
9th	1/1	20
10th	1/1	24
Avg., the first 10:	24/24 (100%)	16 (8–34)
The latest	2/2	10 (9–11)
2nd from the latest	2/2	12 (11–13)
3rd "	2/2	18 (7–29)
4th "	2/3	10 (9–10)
5th "	2/2	11 (11–11)
6th "	1/1	11
7th "	1/1	10
8th "	2/2	11 (10–11)
9th "	1/1	12
10th "	1/1	12
Avg., the latest 10:	16/17 (94.1%)	12 (7–29)

As of March 1, 1955.

tion was carried out in two steps. The first step was an examination made without taking into account the age of tumors. Then a re-examination was made exclusively of four-day-old tumors. In every instance, 100 nuclear plates in each strain were subjected to examination. It was already demonstrated, by the first examination, that each tumor strain had its own mode of variation in the chromosome number, although the fluctuation range was relatively wide. The second examination on tumors of a definite age served to emphasize the above-described individual differences in the mode of the chromosome number (TABLE 12 and CHART 3).

The procedure of the examination may be noted briefly. Staining with aceto-gentian violet solution has been described (*cf.* the second article). In the first examination, the tumor fluid originating from animals bearing tumors of various growth stages (days) was employed without selection for the examination.

In the second examination, four-day-old tumors exclusively were subjected to examination, because it was considered that the tumors were at the height of their growth in this stage, without being accompanied by significant degenerative processes. For the more slowly growing tumors, however, such as AH 601 and 602, four days might be regarded as a somewhat earlier stage of growth. Although usually the ascitic fluid of one tumor animal is of sufficient

TABLE 8

RATE OF TRANSPLANTATION AND SURVIVAL DAYS IN THE FIRST AND THE LATEST 10 GENERATIONS OF SERIAL TRANSPLANTATION

Ascites Hepatoma: Strain 130

Generation	Rate of transplantation "Takes"/animals	Survival days Mean(min.-max.)
1st	3/3	26 (10–43)
2nd	5/5	15 (12–18)
3rd	6/6	16 (15–22)
4th	6/6	16 (11–25)
5th	6/6	15 (10–29)
6th	4/4	14 (12–15)
7th	4/4	18 (12–36)
8th	3/6	23 (13–31)
9th	6/6	26 (12–40)
10th	3/4	25 (13–49)
Avg., the first 10:	46/50 (92.0%)	19 (10–49)
The latest	2/2	11 (9–12)
2nd from the latest	2/2	11 (10–12)
3rd "	2/2	12 (12–12)
4th "	2/2	12 (12–12)
5th "	1/2	12
6th "	2/2	13 (12–13)
7th "	2/2	13 (11–14)
8th "	2/2	11 (10–11)
9th "	4/4	13 (8–18)
10th "	2/2	11 (10–12)
Avg., the latest 10:	21/22 (95.5%)	12 (8–18)

As of March 1, 1955.

TABLE 9

ASCITES HEPATOMA: AH 130

TRANSPLANTATION WITH A SINGLE CELL

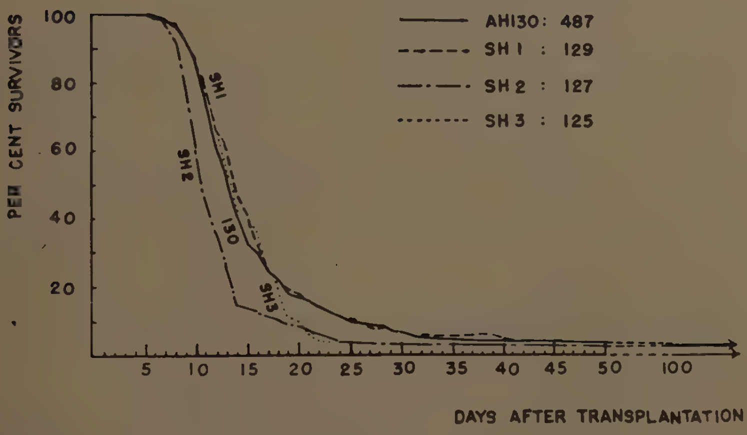
	Number of cases		Result		
	Total	Effective	+	−	%
Intraperitoneal transplantation	123	98	3	95	3.1

TABLE 10

ASCITES HEPATOMA: AH 130 TRANSPLANTATION WITH A SMALL NUMBER OF ISOLATED SINGLE CELLS*

No. of cells transplanted	Result
40	−
26	+
21	+
19	−
2	+

* Intraperitoneal transplantation.



DIAGRAMS OF PERCENTAGE SURVIVAL OF THE 3 CLONAL SUBLINES OF AH130 : SH1, 2 & 3, AND THE MOTHER-LINE AH130.

(JAN. 1955 : SASAKI INSTITUTE)

CHART 2

TABLE 11

CLONAL SUBLINES OF AH 130: SH 1, 2, AND 3

Strain	Established in	Generation	Number of animals transpl.				Rate	Survival days*
			Total	Valid	+	−		
SH 1	Sep. 1953	1–56	130	129	127	2	98.4	14
SH 2	Sep. 1953	1–57	132	127	124	3	97.7	10
SH 3	Oct. 1953	1–53	129	125	122	3	97.6	14

* Median value.
January 1955, Sasaki Institute.

quantity in four days to be used for thorough investigations, the fluid tapped from several animals bearing tumors of the same age may be pooled in a glass tube and mixed with the acetogentian violet solution after the above-described method. The tube, kept at room temperature, can serve as the source of fresh preparations whenever needed.

The transplant generations of each strain at the time of the second examination (TABLE 12 and CHART 3) extended, in AH 130, from the 139th to the 140th; in AH 7974, from the 118th to the 129th; and in both AH 601 and AH 602, the examination was completed in the 60th and 49th generations respectively.

It should be emphasized that all metaphase plates met with under the microscope were examined for their chromosome number. This precaution was considered necessary because it was feared that otherwise those nuclei having a rather small number of chromosomes would be chosen preferentially and the

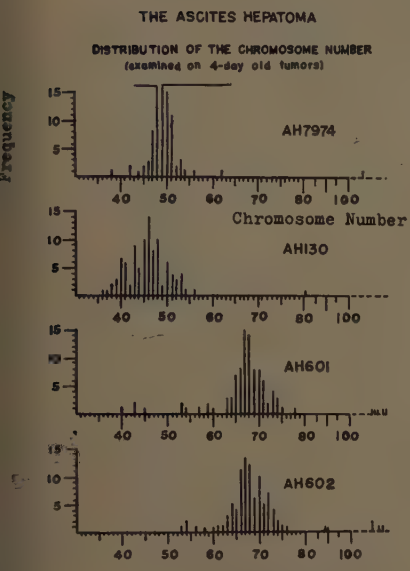
TABLE 12

VARIATIONS OF CHROMOSOME NUMBER OF THE ASCITES HEPATOMA (EXAMINED ON FOUR-DAY-OLD TUMORS) AH 7974, 130, 601, AND 602

No. of Chromosome	Frequency				No. of Chromosome	Frequency			
	AH 7974	AH 130	AH 601	AH 602		AH 7974	AH 130	AH 601	AH 602
36	—	1	—	—	65	—	—	7	4
37	—	1	—	—	66	—	—	8	11
38	1	2	—	—	67	—	—	15	13
39	—	3	—	—	68	—	—	14	12
40	—	7	1	—	69	—	—	9	6
41	—	6	—	—	70	—	—	8	10
42	2	2	—	—	71	—	—	6	5
43	—	9	2	—	72	—	—	2	7
44	1	5	—	—	73	—	—	4	4
45	2	10	1	—	74	—	—	3	2
46	3	14	—	—	75	—	—	1	1
47	8	8	—	—	76	—	—	—	1
48	20	10	—	—	78	—	—	1	—
49	28	2	—	—	81	—	1	—	—
50	15	6	—	—	89	—	—	—	1
51	11	4	—	—	90	—	—	—	1
52	2	3	—	—	—	—	—	—	—
53	3	4	2	1	102	1	—	—	—
54	1	1	1	2	122	—	—	—	2
55	—	—	—	—	123	—	—	1	—
56	1	1	—	1	129	—	—	1	—
57	—	—	1	—	130	—	—	1	1
58	—	—	—	1	132	—	—	—	1
59	—	—	2	—	133	—	—	1	—
60	—	—	1	1	136	—	—	1	—
61	—	—	—	1	141	—	—	—	1
62	1	—	—	1	145	—	—	—	1
63	—	—	3	3					
64	—	—	3	5	Total	100	100	100	100

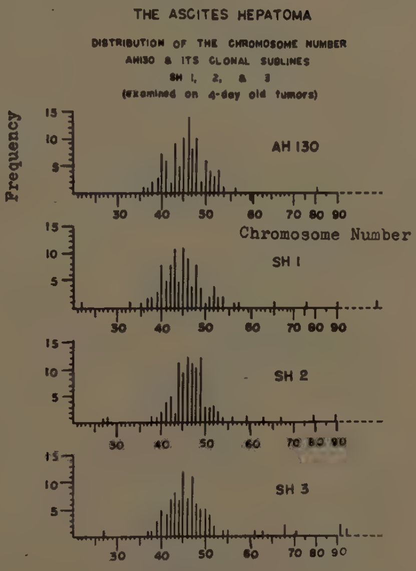
results therefore would deviate from what they actually might be. The chromosomes were drawn, as a rule, with the aid of Abbe's apparatus (FIGURES 12 to 15) and, in cases where the number of chromosomes was not clearly discernible, it was marked, and the question was left open. Thus, for example in a picture showing an indeterminable number of 50 or 51 chromosomes, the result was expressed as 50 + α, where α signifies the ambiguous number. In the present studies a total of 1,100 nuclear plates were examined, and the "α" was used in 73 cases. The value of "α" did not exceed four, with one and two comprising the majority.

During this work, we learned that a hypotonic medium acts favorably on the spreading of chromosomes,[12] and Nakamura introduced the following procedure: 30 cc. of a 0.5 per cent NaCl solution is injected into the abdominal cavity of the tumor animal, which is sacrificed prior to the injection. Immediately thereafter, the opened peritoneal cavity is emptied of fluid (highly diluted tumor ascites), which is centrifuged in a tube. A large amount of acetogentian violet solution is then added to the pellet. The tumor cells in the pellet are



(APRIL 1955 : SASAKI INSTITUTE)

CHART 3



(FEB. 1955 : SASAKI INSTITUTE)

CHART 4

ready for examination as described in the second section of the present paper. The over-all procedure is best completed within 30 minutes.

Excellent pictures, favorable for chromosome counting, are obtained by this method (FIGURES 13 to 15). In the present report, the chromosome number of AH 7974, 601, and 602 (except AH 130) of TABLE 12 and CHART 3 were examined, using this hypotonic method. It is noteworthy that after introducing this method the chromosome number could always be definitely counted in every case in the 300 cells of the above three strains, without need of the "α."

To summarize the results obtained, it is perhaps not surprising that the chromosome number of AH 601 and AH 602, being tumors of the same descent, was identical. Between AH 7974 and AH 130 there was a slight but perhaps significant difference of three in the mode of the chromosome number. Most striking was the fact that the modes of AH 130 and AH 601 (AH 602) differed from each other by about 20. It was further noted that every strain presented only one mode, without suggesting any possibility of polymodal distribution of the chromosome number. These observations may suggest that each tumor, originating from a common normal ancestor, the liver cell, can have its own type of variation of the chromosome number.

In the three one-cell-derived, clonal sublines of AH 130, that is SH 1, 2, and 3, the chromosome number was also examined in the same way as in the second examination, employing four-day-old tumors. The examination was performed in each strain after passage through 41 transplant generations. As shown in CHART 4, the modes of chromosome numbers of the three clonal substrains agreed almost completely with each other, and also with the mode of the mother strain AH 130.

There are investigations that have demonstrated that in sublines of polymodal ascites tumors the mode of the chromosome number differed significantly from the predominant mode of the mother cell population.[16] In thi regard, the conformity of the three clonal substrains to the mother strain, AH 130, demonstrated by the present investigation, may apparently be due to th fact that the mother population of the clones did not show any possibility o polymodal variations of the chromosome number. This suggests, on the othe hand, that the chromosomal type of this kind of tumor is imparted to each and every descendent cell.

It may be added that the examination of the chromosome number of each of the three clonal substrains was performed separately by three different persons, Isaka, Nakamura, and Odashima, and that it illustrates the agreement in their respective results.

The islands never indicate an anomalous and disordered aggregation o tumor cells, but form, instead, clonal groups of cells. The single-cell transplantation may explain the process of island building, but more illustrative i the experiment with inocula treated with trypsin, as Essner, Sato, and Belkin of the National Cancer Institute have first demonstrated. By means o trypsin treatment, the islands of the tumor ascites are disaggregated and abou 99 per cent of the cells become isolated, free cells. Inoculated with such treated tumor fluid, there appear increasing numbers of islands of variou sizes.* Nakamura[24] has been engaged in studies on the differences in th chromosome number among tumor cells within individual islands, the naturally present clones of various sizes. From the results, it became known that even between two cells of a "double" there was a difference in the number of chromosomes, the largest difference noted being six. Rarely was the chromosome number exactly the same in 2 cells belonging to an island, the largest difference so far noted being 39, between 2 cells of a 24-celled island.

Different Responses to Nitrogen Mustard Derivatives by Different Strains of Ascites Hepatoma

In our screening work for cancer chemotherapy, we use mainly the so-called Yoshida sarcoma,[31] and also the ascites hepatoma. In the case of the hepatoma, AH 130 is preferred because of its high transplantability as well as the benefit of cytological examinations, being composed of ample free cells, besides generally small islands. Nitrogen mustard N-oxide, which proved to be most effective on the ascites sarcoma animals, presented about 80 per cent complete cures in cases of animals bearing four-day-old tumors of AH 130. This surpassed by far the therapeutic effect demonstrated on the (Yoshida) ascites sarcoma (CHART 5, A).

It was found in further experiments by Hiroshi Satoh, however, that tumor animals of another ascites hepatoma, AH 7974, were surprisingly resistant to the same compound. Since this was an entirely unexpected observation, the experiment was extended and repeated, using a total of as many as 110 ani-

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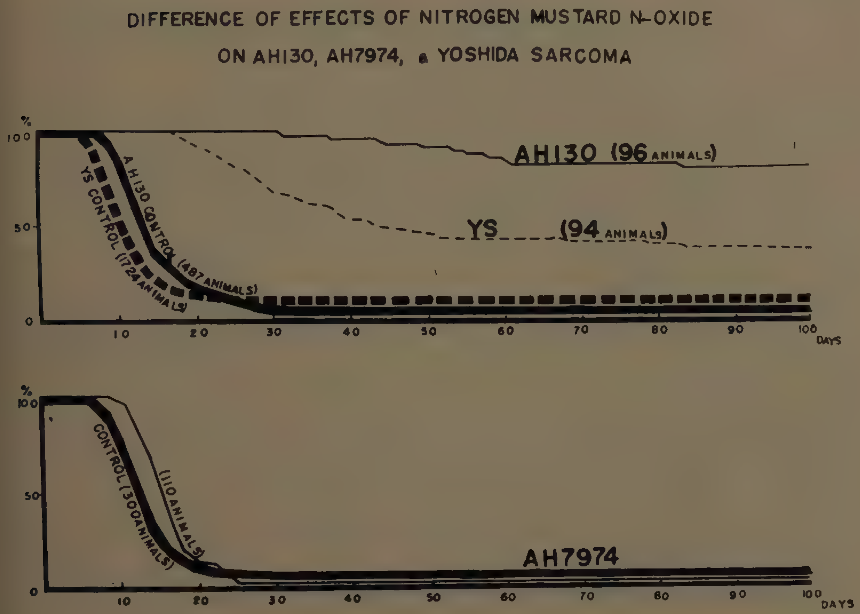
* As to the question of whether every island could actually be traced to a single cell, a careful examination by Sato, Essner, and Belkin will be published soon in Experimental Cell Research, Academic Press, New York, N. Y. (personal communication).



(APRIL 1955 : H. SATOH)

CHART 5

mals, with a result that further confirmed the first observation (CHART 5, B). On the other hand, other nitrogen mustard derivatives, a total of 13 compounds* whose inhibitory effect on the ascites (Yoshida) sarcoma is already known, were tested on both ascites hepatomas, AH 130 and AH 7974, in order to find out the eventual differences in the response of both tumors to these compounds. The treatment with each compound was started in both strains with four-day-old tumor animals. The results are summarized in CHART 6. Thirteen curves indicating the results of all the compounds tested are included in CHART 6, A and B, within the shaded zones of both strains. In AH 130, all the compounds could bring about a complete cure in almost 90 per cent of the animals tested as well as a prolongation of life of the remaining ones (CHART 6, A) while, in AH 7974, all the animals remained completely indifferent to all the compounds (CHART 6, B). It has thus been demonstrated that strain 7974 is completely refractory to all these alkylating agents that had significant inhibitory effects on the ascites hepatoma, AH 130, as well as on the ascites (Yoshida) sarcoma.

Comparative studies with strains AH 601 and AH 602 are not yet complete, because these slowly growing and less transplantable tumors are not themselves

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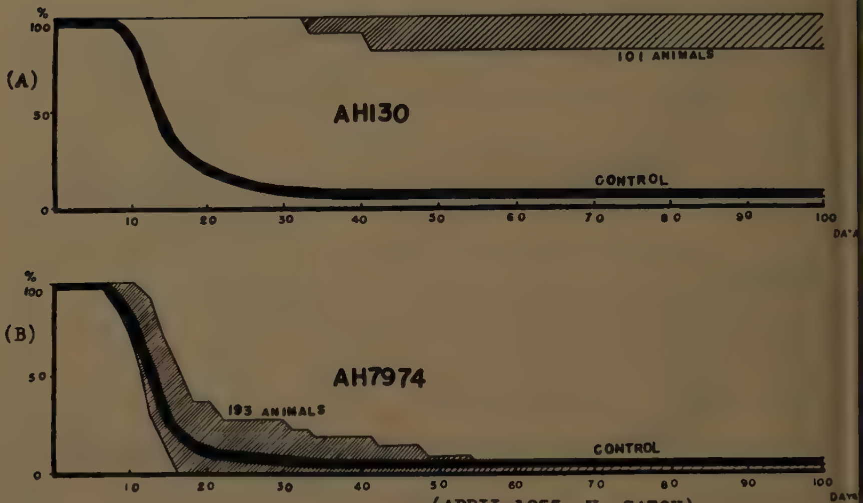
* Methyl-B.C.E.A.; isopropyl-B.C.E.A.-N-oxide; N-propyl-B.C.E.A.-N-oxide; ethoxyethyl-B.C.E.A.-N-oxide; cyclohexyl-B.C.E.A.-N-oxide; allyl-B.C.E.A.-N-oxide; benzyl-B.C.E.A.-N-oxide; benzyl-B.C.E.A.-p-sulfonic acid; p-(naphthol-2-azo-1)benzoyloxyethyl-B.C.E.A.; N-bis(β-chloroethyl)glycine-ethylester-N-oxide N-bis(β-chloroethyl)glycine-N-oxide; N-bis(β-chloroethyl)ananine; N-bis(β-chloroethyl)taurine.

Note. -B.C.E.A. = bis(β-chloroethyl)amine.

DIFFERENCE OF EFFECTS OF VARIOUS NITROGEN MUSTARD DERIVATIVES ON AH130, & AH7974



(APRIL 1955: H. SATOH)

CHART 6

adaptable to the evaluation of the cytostatic effects of chemicals. Examina tions with other kinds of cytostatic compounds are going on, in order to kno whether the unanticipated resistance of AH 7974 to the HN_2 derivatives wa limited to this kind of compound.

As is generally accepted, cancer patients can become resistant to chem therapy by long and repeated treatments with the same drug, as in the ca of infectious diseases, such examples having been reported, especially in th chemotherapeutic treatment of leukemia. On the other hand, information abundant that transplantable animal tumors may acquire resistance to chem cals after continued treatment with the same compound.[2, 8] The resistan to the HN_2 derivatives shown by AH 7974 in the present observation, howeve is by no means an induced resistance, but rather a natural one. Further i vestigation is needed for an understanding of the biochemical nature of th resistance.

The observation deserves a brief description relating to the current discussio on the principle of screening work for cancer chemotherapy. An advanta of the "tumor spectrum" in screening work is found in the wide variety tumors that are expected to show different responses to a chemical compoun under test according to the difference in their innate nature, which, in tur is accepted as signifying the difference of the normal mother tissues from whic they have originated. However, the fact that two tumors of common norma ancestry can respond to the same compound in entirely different ways seem to be of significance in the discussion of the principle of the screening work as well as in the evaluation of clinical effects of chemicals on human cancer, which can vary even in the same kind of tumor of the same organ.

Discussion: Concept of the Malignancy of Cancer

The concept of the malignancy of cancer is formed only in relation to the host. Microscopic pictures may visualize the malignancy of cancer where tumor cells are invading the normal tissues, and thereby may arise the concept of the grade of malignancy but, when one observes a droplet of tumor ascites under the microscope, only cells are found, living their own silent lives, like protozoa, suggesting nothing about malignancy, much less its grade. The only thing perceivable is the rapidity of growth, which is implied in the plentiful number of mitoses present.

When malignancy is examined in a measurable way, two qualities of the tumor, at least, may account for this concept: the growth rate and the range of transplantability of the tumor. From this point of view, a grading of malignancy may first be realized. Then, the next question of interest is whether such a grade of malignancy would change, especially increase, within an individual tumor, hand in hand with the progress of its growth. In our previous studies with an ascites sarcoma, the so-called Yoshida sarcoma, an increase, especially a gradual increase, in malignancy was not observed, and it remained stable from the onset of the tumor throughout further transfer generations, extending over a period of nearly 10 years.[33]

In the present studies with the ascites hepatoma, we paid special attention to this kind of problem. We employed four strains (AH 130, AH 7974, AH 601, AH 602) of ascites tumors for the examination, but they are actually regarded as three strains, since AH 601 and 602 comprise two sublines from the same hepatoma.

It has been demonstrated that both qualities of tumors, the growth-tempo and the transplantation rate, remained unchanged in each strain during the term of observation extending from the onset to over a period of three to four years. Each of the four strains presented its own microscopic picture of the tumor ascites, which differed significantly from each other, so that the picture was characteristic for each strain.

Three one-cell-derived, clonal sublines of AH 130 have been kept for more than 50 transfer generations, and they have demonstrated a conformity to the mother strain, AH 130, in all of the aspects examined.

As to the mode of the chromosome number of each strain, a slight difference of about three was noted between AH 130 and AH 7974, while the difference between AH 130 and AH 601 (and 602) was as much as 20. Each strain presented one-mode variations, without suggesting the occurrence of admixed cell populations of significantly different chromosome numbers or ploidy within the predominant mode. The mode of the chromosome number of three single-cell-derived, clonal sublines of AH 130, examined after passage through more than 40 transfer generations each, agreed almost completely with each other, and with that of the mother line AH 130.

These observations have given us the impression that every tumor has its own individual character or its own "grade" of malignancy, which is stable and distinguishable even among tumors of common normal ancestry. Observations on the clonal sublines may suggest that the individuality of the tumor is imparted to each and every descendent cell.

On the other hand, there was the observation that two ascites hepatoma strains of different origins, AH 130 and 7974, being closely similar in all other aspects examined, responded to the treatment with HN_2 derivatives in a completely different manner. This may provide another clue in identifying the individuality of the tumor.

It is a classical question of cancer pathology, whether the clinical grading of the malignancy of cancer which, as is generally accepted, can increase gradually in one and the same patient, is necessarily connected with biological changes in the cancer cells themselves. The results of the present study have led us to the belief that the cause of "clinical increase" in the grade of malignancy, or the increasingly malignant appearance of tumors, either in gross or microscopic pictures, should be looked for in changes of the environmental condition of tumor cells or in the general conditions of the patient rather than in biological changes of the tumor cells themselves.

In this connection, a general preference for the word "anaplasia" to "degeneration" of tumor cells in tumor pathology may deserve a brief discussion. In the tumor ascites of the end stage, there appears an evident change in the appearance of the majority of tumor cells which is likely to be called "anaplastic" change, but which disappears when this ascites is inoculated into the normal animal and the growth begins there anew, presenting tumor cells of uniform size and appearance. The morphology of the tumor ascites that continues its growth as long as the animal passages last has to be described as a cycle of these alternating changes. The changes of cells in the end stage of one tumor animal are, no doubt, of a degenerative nature due to the changes in the environmental conditions caused by the overpopulation of tumor cells, as well as by the changed general conditions of the host. This kind of change, however, especially when it is observed within the limitation of the patient's body, is often attributed to "anaplasia" of tumor cells, which has an implication of hereditary, biological cell changes.

Stress should be laid, however, on the fact that the main concern of the present discussion is the pathological or medical understanding of cancer, where neoplastic growth is observed within a limited duration of the individual human and animal life, especially under influences of various factors, including occasional infections with microorganisms. In other words, in the interpretation of cellular changes during tumor growth, a distinction between "degenerative" and "biological" hereditary cell changes is required. The latter term is preferable in the field of biological studies of tumor cells, as the work contributed by Hauschka, Klein, Levan, and others to this monograph illustrates.

That the present strains of ascites hepatoma did not change in growth pattern and transplantability might find an interpretation, at least with respect to AH 130 and 7974, in their great rapidity of growth and wide range of transplantability. The two strains are supplied from the beginning with a short survival time and with a transplantability as high as 96 per cent or more in heterozygous animals, and these may be regarded as practically maximum, so that there hardly remains a possibility for further increase. In this relation, the remaining two strains, AH 601 and AH 602, which are growing rather slowly and have a narrow range of transplantability, might have the possibility of changing in the future, and for this reason we are interested in continuing the present observations.

Summary

The ascites hepatoma, prepared by ascitic conversion of the azo-dye hepatoma of the rat, can provide a series of ascites tumors for comparative studies on a variety of tumors of a common normal ancestral cell.

In four different strains of ascites hepatomas, the growth pattern, transplantation rate, microscopic picture of the tumor ascites, and the mode of the chromosome number were studied and compared with each other. Results obtained suggested that each tumor has its own individuality, as far as the above qualities are concerned. These are regarded as fixed from their onset, remaining unchanged throughout 50 to 150 generations of serial transplantations extending over 3 to 4 years.

From one of the four strains, three clonal substrains were successfully established by single-cell transplantations. The growth pattern, transplantation rate, picture of the ascitic fluid, and the mode of the chromosome number, examined after 41 transfer generations, indicated that all the clonal sublines agreed completely with each other, being identical with that of the mother strain. This may suggest that the individuality of the tumor is imparted to each and every descendent cell.

It was noted, on the other hand, that among the ascites hepatomas there were two strains that, being closely similar to each other in all aspects, responded to HN_2 derivatives in completely different ways. One was highly sensitive, and the other surprisingly resistant to the same treatment. The resistance was never acquired by preceding treatments, but was a natural one. The observation may provide another clue in identifying the individuality of the tumor.

The malignancy of the tumor, accepted in a measurable way, is manifested in the growth tempo and transplantation rate, which, as observed above, are unchangeable qualities of the tumor. Therefore, an increase in the grade of malignancy within one and the same tumor is hardly acceptable, but is recognized, instead, as a differing quality among different tumors that can be of common normal ancestry.

Stress is laid on the fact that the main concern of the present discussion is the pathological or medical understanding of the neoplastic growth, where the tumor is observed within a limited duration of individual human or animal life under influences of various possible factors. In view of the present observations, the interpretation of cellular changes that occur during tumor growth necessitates a distinction between "degenerative" and "biological" hereditary cell changes.

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PLATES

PLATE 1

FIGURE 1. Flat and elongated surface cells of a small island. (AH 7974)

FIGURE 2. Phase-contrast microscopic picture of a "double"; one looks like an endothelial cell and the other is in mitosis. (AH 130).

FIGURE 3. Beadlike association of tumor cells, about 2 weeks after inoculation with a single cell. (AH 130).

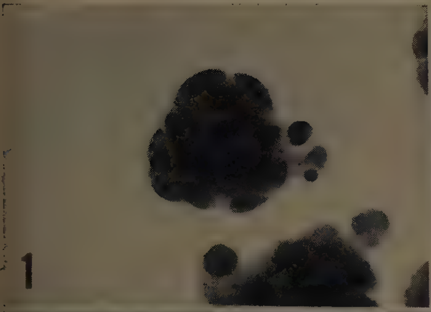
FIGURE 4. Endothelial-like appearances of tumor cells; left: phase-microscopic view; right: aceto-gentian violet staining. (AH 7974).

FIGURE 5. General view of the tumor ascites of strain AH 601: generally large and compact islands with smooth surface.

FIGURE 6. General view of the tumor ascites of strain AH 602: large, but irregularly shaped islands of loose cell bonding.

FIGURE 7. General view of the tumor ascites of strain AH 7974: uniformly small islands, a fair amount of isolated cells, doubles, triples, and the like.

FIGURE 8. General view of the tumor ascites of strain AH 130: abundant isolated cells among smaller and fewer islands, and looking, in part, like an ascites sarcoma.



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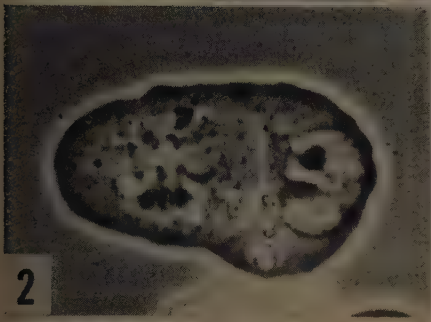

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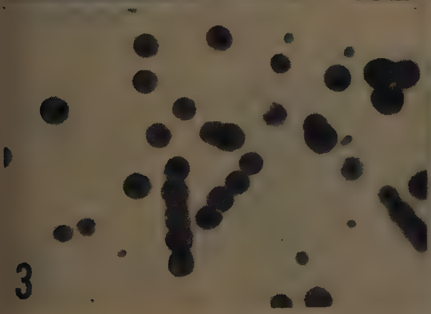

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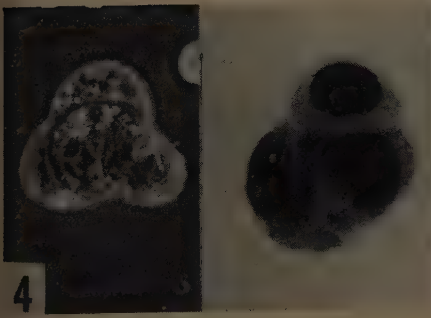

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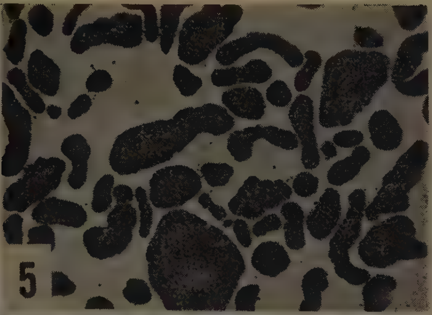

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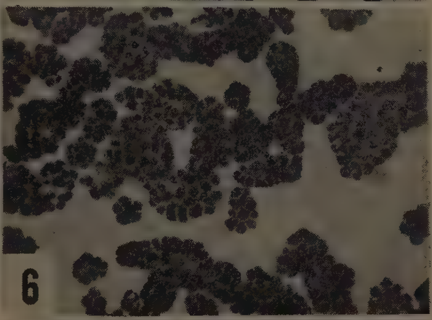

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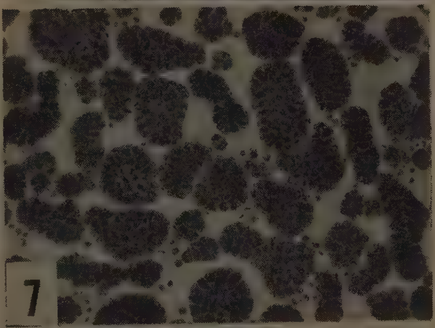

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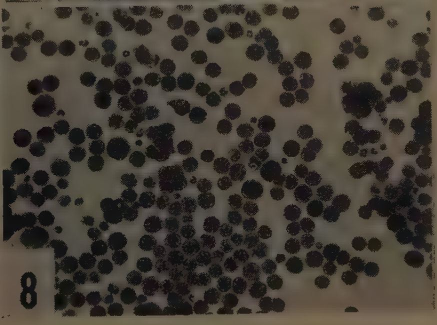

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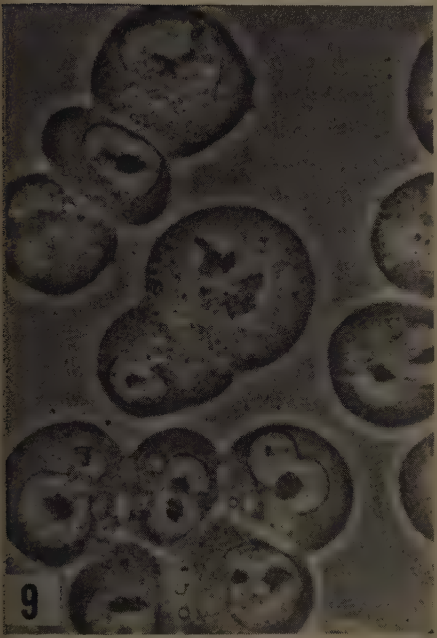
PLATE 2

FIGURE 9. Phase-contrast microscopic pictures of a double and three- and five-celled islands. One cell of the double is in mitosis. (AH 130).

FIGURE 10. Phase-contrast microscopic pictures of a variety of doubles and isolated tumor cells. (AH 130).

FIGURE 11. Numerous hepatoma nodules in the abdomen of a 13-day-old tumor animal of AH 7974, after removing the tumor fluid.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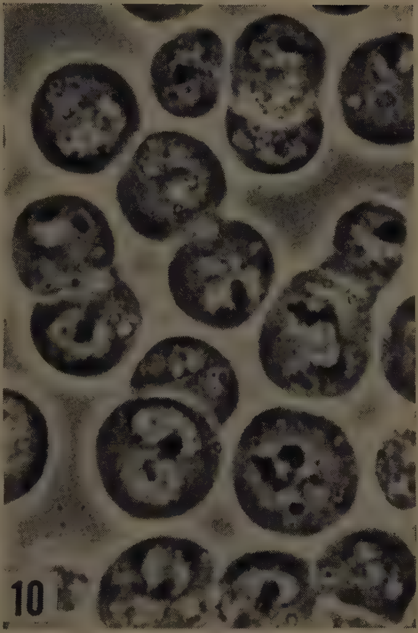
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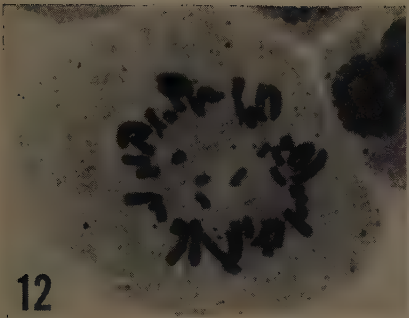
PLATE 3

An example of photomicrographs (on the left side) and drawings (on the right side) of metaphase plates of ascites hepatoma cells.

FIGURE 12. AH 130, chromosome number 49 (without using the hypotonic method).
FIGURE 13. AH 7974, chromosome number 49 (using the hypotonic method).
FIGURE 14. AH 601, chromosome number 67 (using the hypotonic method).
FIGURE 15. AH 602, chromosome number 67 (using the hypotonic method).



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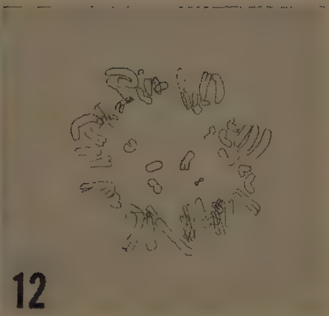

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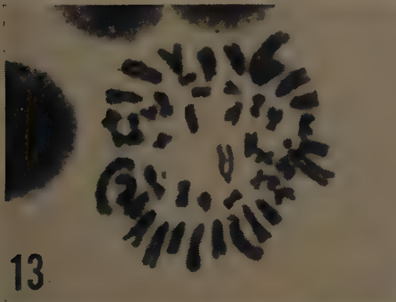

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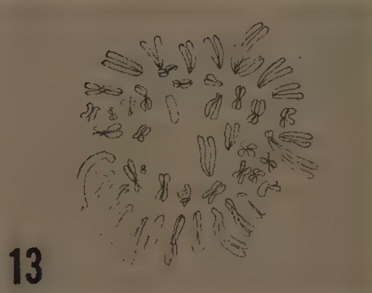

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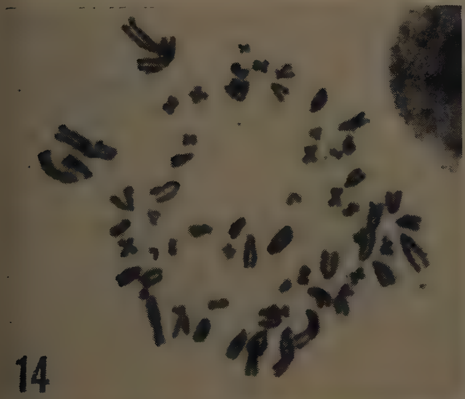

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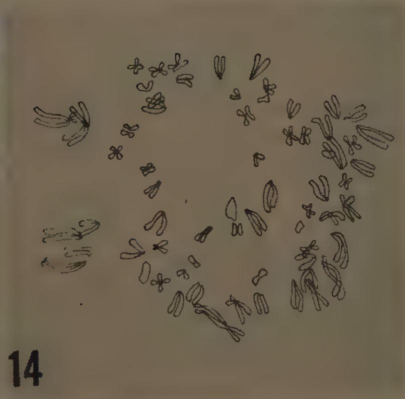

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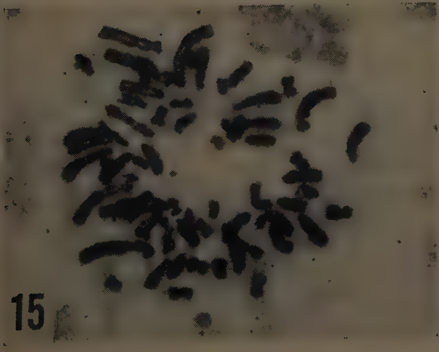

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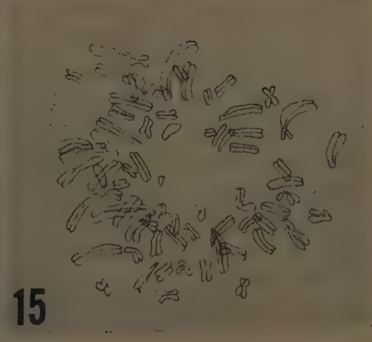

15

THE VALUE OF ASCITES TUMORS IN PROBLEMS OF TUMOR IMMUNITY

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It was believed at one time that transplantation immunity was entirely distinct from any other type of immune response—a somewhat strange conception, for it is difficult to see why some special immune mechanism should be evolved for the benefit of 20th century scientists. Today, few if any hold such beliefs. It is insufficiently realized, however, that we are dealing with material that is very different from that used in classical immunology. We are primarily concerned with such entities as full-thickness skin grafts or solid tumors, and not with freely dispersed bacteria or substances in solution.

Much elegant work has been done with skin homografts, but such homografts are not very suitable objects for antibody detection, the viable epidermal cells being protected on one side by keratin and on the other by dermis. Sarcomata are even less suitable for antibody detection, since they are almost invariably necrotic in the center, and the amount of antigenic material present cannot be accurately assessed.

Epidermal cells can be dissociated by tryptic digestion. This might also be done with some tumors, except that these are generally dissociated mechanically. While such dissociation may not be difficult, there is always a great deal of cellular debris, and any exact estimate of antigenic material is impossible.

Ascites tumors are free from necrotic material, the cells of many are freely dissociated, and the only common contaminant is blood. The blood presents no great problem, however, and can be eliminated if desired.

Before describing data obtained with such material, it is desirable to review briefly certain aspects of the homograft reaction. First, the orthotopic skin graft undergoes no necrotic changes, and there is a minimal reaction following operational trauma. Later, an inflammatory reaction develops, the dermis becomes infiltrated with leukocytes, and the graft is destroyed.

In the case of subcutaneous grafts of sarcomata, the initial picture is somewhat different. Owing to central necrosis, there may be a fairly pronounced polymorph reaction 24 hours after transplantation. This usually subsides, and the subsequent events are not dissimilar from those occurring in skin grafts. It is usual to find lymphocytes in intimate contact with the grafted cells. Lastly, there is the question of leukotic cells. As Potter and Findley[29] first showed, these may be completely destroyed prior to leukocytic invasion. It will be necessary to bear these histological features in mind when discussing the nature of the immune response.

A sarcoma may have some inherent irritative properties, but an orthotopic skin graft does not, and perhaps this is why Medawar was the first to draw analogies between the homograft reaction and the "tuberculin" type of allergy. Allergic reactions are the result of the union of antigen and antibody on the cell surface. Antibodies vary in their affinities for the tissues of the individual

TABLE 1
TYPES OF ANTIBODY AS RELATED TO ALLERGY

Affinity for tissues	Affinity for antigen when free	Examples
0	+++	Cooke's blocking antibody ? isoantibodies.
+	+++	Common. Ordinary precipitating antibodies *etc.*
+++	±	Reagins
++++	?	(?) Favors plasma factor.

forming them, and TABLE 1, which must be regarded as diagrammatic, shows certain types that may occur. Probably most antibodies have some affinity for tissues. This is shown to an extreme degree by the reagins, which are thermolabile antibodies found in sufferers from hay fever, *etc.* These reagins have a very low affinity for antigen when unattached to cells. Cooke and his co-workers[10] showed that if people with reagins were hyperimmunized they might develop a thermostable antibody that would combine with antigen when free and thus block the reactions with sessile reagins.

It is likely that Cole and Favour[9] have shown how to transmit the tuberculin type of allergy by a soluble antibody, but previously this had been accomplished only by living lymphocytes. The picture is not greatly changed, however, by Cole and Favour's exciting discovery. The antibody is assumed to become fixed very rapidly to other types of cells which would then react with tuberculin or the PPD of Seibert. It is important to emphasize two things. First, that more than one type of antibody may be formed against the same antigen and may coexist in the same individual. Dienes[11] and later Chase[8] showed that the tuberculin type of response precedes the appearance of ordinary circulating antibody in those cases where both are formed. Second, it must be emphasized that an allergic reaction results in the death of the host's cells. If, as is quite likely, the lymphocytes one sees in contact with grafted sarcoma cells are transferring the Cole-Favour type of antibody to the malignant cells, one has to inquire how they survived to make the journey. One possibility, which will be explored later, is that they are protected by the presence of circulating isoantibody, which has been classified, therefore, with Cooke's blocking antibody.

Antibody Avoidance and Detection

The most common cause of failure to detect antibody arises from the widespread belief that if antibody is present it must be so in high concentration following regression of a single graft. This is by no means always the case, and any critical search should involve the use of hyperimmunized animals. Failure to do so led to the failure of Todd and Kidd[33] to detect a cytotoxic antibody against the lymphosarcoma 6C3HED. The same type of error occurred in the work of Gaudino[12] and of Prehn, Weaver, and Algire,[30] although in both these cases there were additional reasons for failure. In all these cases, antibodies have been detected by other workers.

The most important isoantigenic system for tumor inoculation in mice is

TABLE 2

SYSTEMS FOR DETECTING HEMAGGLUTININS IN MICE

Cells suspended in:	Antibody diluted in:
Normal human serum	2% Intradex
Normal human serum	Ovarian cyst fluid
Myeloma serum	Saline
Normal human serum	U. S. dextran No. 2058
Normal human serum + globulin	Saline
Normal human serum	Normal human serum
Normal human serum	Saline
*Unheated human serum	Saline
Mouse serum	Mouse serum
†2% Mouse serum	Saline

* Gives hemolysis.

† Any serum may be used. Often gives much higher titers if spun. From Gorer,[13–16] and Gorer and Mikulska.[17]

the H-2 system of antigens. Most of these have been detected by hemagglutination techniques by ourselves and by Hoecker, Counce, and Smith.[18] The various ways in which hemagglutinin reactions may be obtained are shown in TABLE 2 in ascending order of efficacy. For those systems shown below the line, the order is only approximate. The least efficient system, shown at the bottom of the TABLE, is generally referred to as the "saline" system. Occasionally this system may succeed when the human serum plus globulin fails, so that the order of efficiency below the line is only approximate. The systems shown above the line are approximately coequal in efficiency. Of these, we now use the human serum-dextran system exclusively, and it will be convenient, in future discussion, to refer to the dextran titer as representative of all those above the line. The dextran titers are usually much higher than the systems below the line, and also much more stable. The various types of activity may appear and disappear on storage (this is seldom true of the dextran titer but not infrequent in the others). It seems probable that these reversible changes reflect changes in the physical state of the antibody molecules. We have no information as to which physical state is likely to be most efficacious *in vivo*. This state may or may not correspond to that giving the best results *in vitro*.

In TABLE 3 are shown the known H-2 antigens of the stocks used in our own experiments. Other combinations were studied by Hoecker, Counce, and Smith,[18] who discovered the C antigen. Snell and his colleagues used the orthodox genetical symbols H-2^{d} *etc*., to describe the alleles. For the investigator mainly interested in the antigenic components, it is convenient to use a phenotype symbol H-2D *etc*. Genetically, the system is very peculiar. Mutations from H-2^{b} to H-2^{d} have apparently occurred more than once in the C57B1 strain. It will be seen from the table that this involves loss of C, B, and E with the appearance of C, D, and E^{d}. We have observed, however, two crossovers—the first between B and E, and the second between D and K—both of which are involved in the mutational change. The new combination,

TABLE 3*

THE H-2 ANTIGENS IN FOUR STRAINS OF MICE AND COMBINATIONS FORMED BY CROSSING OVER

Strain	Genotype symbol	Plentotype symbol	Known antigens
A	H-2^{a}	H-2A	CDEFK
Balb. C	H-2^{d}	H-2D	CDE^{d}Fk
C$_3$H	H-2^{k}	H-2K	CdEfK
C57 B1	H-2^{b}	H-2B	c“B”EFk
	Crossover between “B” and E		?BE^{d}Fk
	Crossover between D and K		CBEFK

* From Amos, Gorer, and Mikulska.[2]

so formed, is known to have the antigenic composition CBEFK. It also shows that B is an allel of D, and the terminology should be changed accordingly.

These antigens are sometimes described as blood-group antigens. This is unfortunate, as they are predominantly tissue antigens, being most abundant in tumors, spleen, lymph nodes, liver, and mammary glands, less so in kidney and salivary gland, and poorly represented in muscle. The antigen in muscle has not been compared directly with blood, but one absorption with liver has about the same effect in removing antibody as five absorptions with red cells. A very interesting situation occurs with regard to antigen E. This antigen is present in easily detectible amounts on the red cells of strain A and C3H, but in very small amounts on the red cells of strain C57B1. Indeed, for some time we doubted that it was present at all in the latter case, and we had to use special methods to detect it. This antigen, however, is well represented on C57B1 liver *etc.* All of these antigens are present on leukocytes, and Amos (unpublished) has evidence that some components may be absent from red cells but present on leukocytes. One such possibility is *k*, which may be shared by the H-2B and H-2D combinations. It will be remembered that H-2^{a} used to be called H-2^{dk}, because crosses between mice of types H-2D and H-2K were susceptible to A-strain tumors. Serology showed that the situation was more complex, and the terminology was changed. It will be seen that the former A H-2 combination supplies antigens C, D, and F, and the latter, C, E, and K. This fact, together with the known abundance of the antigens on tumors, *etc.*, gives us confidence that these components are of functional importance.

The Action of Antibodies on Leukotic Cells

In branches of immunology other than transplantation immunity it is customary to attempt to titrate sera. There are two methods of titration employable with mammalian cells. One method is to keep the quantity of antigen constant and vary the amount of antibody. The ordinary hemagglutinin titration is a constant antigen titration. When studying the direct actions of antibodies on malignant cells in mice, however, it is generally more convenient to use a constant antibody titration. The titer of the serum can then be expressed in the number of cells affected. The choice here is partly dictated by the nature of the material being used. With an ascites tumor, one animal can give enough cells for any experiment of practical size, while the amount of serum obtained from a single mouse will seldom be more than 0.5 ml., and often less.

In theory, there are three ways of testing the effects of antibodies on cells:

(1) The cells may be exposed to the action of antibody plus complement. This method has been successfully performed by Kalfayan and Kidd[23] with the Brown Pearce tumor in the rabbit. Unfortunately, mouse serum has very peculiar properties. It is allegedly deficient in one of the components of complement.[6, 24, 31, 32] Furthermore, isoimmune sera are very often strongly anticomplementary. So far, no one has succeeded in finding a really satisfactory method of complementing the system even with red cells, although occasional successes have been obtained here (see TABLE 2 and Gorer[16]).

(2) Cells are mixed with serum prior to inoculation into suitable hosts. We have done this successfully with leukotic cells with two different leukoses, one arising in the A strain,[14] and one chemically induced in C57 BL, known as E.L.4, now maintained as an ascites tumor, which will be referred to below. This experiment has been independently confirmed by Mitchison,[26] using the oestrogen-induced 6C3HED. Burmester[7] obtained similar results with a fowl leukosis and, finally, Billingham and Sparrow[3] performed the notable technical feat of demonstrating a cytotoxic effect on dissociated epidermal cells in the rabbit.

Burmester, Billingham, and Sparrow found that prolonged incubation was needed prior to inoculation. This is not essential with mouse leukoses, but its effect on titer has not been adequately studied. Expressed in terms of cells inhibited, the latter varies from about 10 thousand to 1 million cells subsequently inoculated into the susceptible strain. Higher figures might be obtained if resistant mice were inoculated.

(3) We may refer to this type of experiment as a neutralization technique, to distinguish it from effects observed when cells and antibody first meet *in vivo*.

For work of this kind, E.L.4 presents extremely favorable material. Moderate cell doses injected subcutaneously into mice of alien strains (A, Balb. C and C3H) give rise to a tumor that becomes palpable about the fourth day and has a mean diameter of about 1 cm. by the 7th or 8th day. It then regresses rapidly and disappears between the 10th and 14th days.

Our sera were from mice that had been hyperimmunized, and took from six to eight weeks to prepare. Preliminary work on the survival of antibodies was done by Amos,[1] who studied the behavior of hemagglutinins when serum was injected into mice of suitable antigenic type Amos also performed a few neutralization tests and found that the antibodies were still active after three days' residence in a secondary host.

Together, we have performed a number of experiments on passive immunity to E.L.4. In most cases, it was possible to suppress the growth entirely. The titer of sera varies between about 500,000 and 3 million cells (the latter is probably exceptional).

TABLE 4

EMONSTRATION OF ANTI-X ANTI-E.L.4. 800,000 E.L.4 INOCULATED SUBCUTANEOUSLY

ouse of strain and No.*	Mean diameter in mm. of tumor in controls on day No.†			Strain No.	Mean diameter in mm. of tumor in experimental group on day No.		
	6	8	13		6	8	13
A.1	14.5	12	+	A.5	—	—	—
A.2	13	10.5	+	A.6	—	—	—
A.3	12.5	10	9	A.7	—	—	—
A.4	12.5	10.5	+	A.8	—	—	—
C57 B1 1	12	14.5	20	C57 B1 5	—	—	— All killed with large tumors on 30th day.
C57 B1 2	11.5	12.5	17	C57 B1 6	—	—	—
C57 B1 3	13	14.5	17	C57 B1 7	—	—	—
C57 B1 4	11	12	16.5	C57 B1 8	—	—	—

Symbols such as + mean that a tumor is definitely palpable but too small to measure. tr = very small gran-
e. From Amos and Gorer (to be published).
* Numbers are arbitrary.
† Controls usually die about 21st day.

We then wished to examine the relative values of various H-2 components, ut found that, *in vitro*, we were apt to get nonspecific absorption. We there- re decided to absorb *in vivo*. To our great surprise, protective action was ill found after antibody had been 24 hours or more in C57 B1. We decided) call this component the *X* component, and it will be discussed again later ee TABLE 4). As was pointed out earlier, leukoses show a very unusual ehavior when regressing, and we naturally wondered whether the unexpected se with which we had obtained passive immunity was due to some peculiar roperty of the *X* component and anti-*X*. We prepared sera against a C57 B1 ammary carcinoma of recent origin and against normal C57 B1 tissues in alb C. mice. Both of these protected Balb C. mice, but not C57 B1, although here was a slight suggestion of some effect in the serum produced against ormal tissues.

The most obvious explanation of the *X* component is that a mutation has ccurred in some H locus, either in E.L.4 or in C57 B1. The latter is more kely, since selection would have to be heavily in favor of newly arisen in- mpatible cells for them to become established. However this may be, anti-*X* obviously a potent antibody and we must assume *X* to be a good antigen. e should, therefore, find it easy to immunize C57 B1 mice or F_1 mice against .L.4. So far we have failed. When a subcutaneous growth becomes large ough to ligate, it has already spread into underlying muscles or regional mph nodes. Inoculation into the tail has given three types of result: (1)) local lesions and no immunity; (2) no local lesions with death from internal sions; and (3) a local lesion with death from internal lesions. The last has ccurred—even when the tail has been amputated—with a very small tumor. egative evidence is always unsatisfactory, and it seems to us that it is desirable) know if E.L.4 is a special case, or whether an *X* component is a common ature of leukemias.

Antibody Effects on Sarcoma Cells

Experiments of the neutralization type have been performed by myself an independently, by Mitchison and Dube.[28] If the cells were reimplanted in the susceptible strain after exposure to antibody, we both found the rate growth to be accelerated. If the cells were reimplanted into resistant mi the growths might be partially or completely inhibited.

Passive immunity experiments, however, gave a somewhat surprising resu Kaliss[19-22] and various of his colleagues found that growth was enhanced, did Mitchison and Dube. Indeed, Kaliss and Molomut[21] found that rab antimouse serum would enhance.

The most obvious explanation of this surprising result might appear to that such small amounts of antibody as reached the sarcoma cells simp served to stimulate them to divide. It is possible that this accelerant eff plays some part, but it does not appear to be the main factor.

The experiments to be described were done with a C3H ascites sarcom B.P.8 given me by Doctor Craigie. This sarcoma is a highly polymorph growth and perhaps is not ideal for this type of work.

In TABLE 5 are shown some experiments with a sample of Balb. C anti-B.P. I wished to see whether a mild inflammatory reaction, artificially induce would influence the effect the antibody. The first experiment (not shown the table) in which mouse serum was used as a vehicle gave a suggestion some retardation of the growth, while regression failed to occur in a major of the mice. When normal human serum was used as a vehicle, it could seen that very considerable retardation occurred, but that regression was hibited. When washed human red cells were added, the retardation mig be greater and the growth entirely suppressed. In one case, a very sm growth occurred which rapidly regressed. The same experiment was perform using mice that had been actively immunized against human blood. Seru produced no retarding effect in this case, but regression was inhibited by t isoantibody and, to some extent, even in the controls. The most compl suppression of growth to be observed, thus far, has been obtained with anti-B.P.8 and cells suspended in normal mouse serum and killed My. tub culosis. Sera from less well immunized mice give very little retardation growth and a very minor delay in regression.

It is apparent that acceleration of growth rate is not operative here. appears that sarcoma cells are effectively destroyed only when infiltrated wi leukocytes. For this to occur, a sustained inflammatory response is need and antibody is unable to elicit this. Indeed, as Amos[1] has shown in expe ments with E.L.4, very little antibody reaches a subcutaneous growth, exce perhaps just before regression begins. The bulk of it, therefore, is in the tiss spaces, *etc.*, and it seems quite likely that antibody is combining with antig in these situations and reducing the amount reaching the lymph nodes, splee *etc.*, and thus delaying the allergic response of the host. Indeed, Mitchis and Dube consider that the enhancing effects of lyophilized tissues are due an anergic response. The term "anergy" may denote failure of allergy develop, or its masking by circulating antibody or other agency. Accordi

TABLE 5

THE VARIED EFFECTS OF ANTIBODY ON THE C_3H SARCOMA B.P.8

Serum: Balb. C anti-B.P.8
Dose: 950,000 cells in normal human serum
Hosts: Balb. C

ouse No.	Mean diameter (mm.) of control tumors on day No.				Mouse No.	Mean diameter (mm.) of experimental tumors on day No.			
	5	7	10	21		5	7	10	21
1	7	+	—	—	5	5	7	11	10.5
2	6.5	++	—	—	6	?	9	tr	23†
3	6.5	tr	—	—	7	?	?	?	10
4	+	—	—	—	8	—	—	—	24†

Serum and hosts as above
Dose: 1.1 million cells in mouse serum +3% human red cells.

ouse No.	Mean diameter of control tumors on day No.			Mouse No.	Mean diameter of experimental tumor on day No.		
	6	10	21		6	10	21
9	7	tr	—	13	6	8.5	tr
10	7	tr	—	14	tr	++	14
11	5	tr	—	15	tr	tr	—
12	+	?	—	16	—	—	—

† Two tumors.

Mitchison and Dubo, allergy fails to develop following pretreatment with ophilized tissues, *etc.* Kaliss[19] suggests that circulating antibody is re- onsible for enhanced growth under these conditions. Under suitable nditions, cortisone will inhibit antibody production and abolish the en- ncing effects of pretreatment.[19-20] This is quite consistent with the results tained with "passive immunity" against sarcomata. None of these authors ggests the role played by these antibodies during the acquisition of immunity in the actively immunized host where titers may be extremely high, together th a very high degree of immunity. It would appear that their activity in anergic host must be very different from that occurring in the presence of a stained inflammatory response.

With normal tissue homografts, information is more difficult to obtain. octor Billingham informs me, however, that delay in the regression of a skin mograft has been obtained using large amounts of serum. What is ap- rently an anergic antibody response has been obtained by Bonfiglio, Jeter, d Smith.[5] These investigators made an extract of rabbit bone that elicited mplement-fixing antibody. They observed a delayed inflammatory response subsequent homografting. This is quite consistent with the view that this tibody is reducing the amount of antigen reaching the reticuloendothelial stem.

It is not possible to do justice to the work of Billingham, Brent, and Medawar[4] on the transfer of immunity to skin homografts by means of lymph no and spleen. These authors point out that it is improper to refer to pass immunity in their experiments, since the animals receiving immune lym nodes gave the same type of response as an actively immunized animal. T is quite different from the passive immunity to E.L.4, since the mice are fu susceptible to subsequent inoculation if the growth has been completely s pressed. These workers, therefore, coined the term "adoptive immunit Adoptive immunity could be transferred only by draining lymph nodes spleen. The ability to confer adoptive immunity, however, lasts a considera time, and was successfully performed 30 days after regression of a graft.

While this work is quite consistent with the idea that sensitivity of a dela type is being transferred, the evidence of Mitchison[27] and Mitchison and Dub is more convincing. Most of the work done by these investigators was w the A strain sarcoma Sa.1 in C57 Br/a mice. The ability to confer adopt immunity is not so long-lived as with skin. Transfer was successful betw the 5th and 10th days after primary inoculation. Following a second challen transfer was successful at two days, but faded very rapidly. Transferred no also gave rise to hemagglutinins in secondary hosts. The titers were lower w 5th-day nodes than with 14-day nodes, which is an inverse relationship to power to transfer adoptive immunity. It would appear that the latter depe upon the ability to transfer allergy, and the maximum ability to do thi prior to the time when circulating antibody is being elaborated and, it will noticed, is not closely correlated with the immune state of the donor.

These experiments have been interpreted as indicating that the hemagg tinins are by-products. In my opinion, they indicate precisely the reverse

If one is going to invoke a tuberculin-type sensitivity, one should be pared to follow the logical consequences. The effects of PPD on sensiti cells are well known. The cells may be killed on contact, their migration m be inhibited, and phagocytic powers diminished. The results are, in fa entirely disadvantageous to the host. One must assume that this allergy primarily directed against the H-2 antigens, as are the hemagglutinins. may envisage that the latter will reduce, as they are formed, the antigen c centration in the region of the graft and allow lymphocytic immigration to ta place. It seems very likely that lymphocytes elaborating the Cole-Fav type of antibody are of major importance in destroying sarcoma cells. time of release of hemagglutinin into the circulation is "classical," that is say, it occurs at the time of disappearance of antigen. The titer may somewhat thereafter, which again is "classical," the assumption (outs homografting circles) being that, until the amount of antigen has been s stantially reduced, antibody is being rapidly used up. In general, the prim response to homografts seems to correspond well with the ideas of Dienes,[1] that it is first a "tuberculin" type of response closely followed by a circulat antibody as immunity develops.

We have seen what antibody may do in the anergic host and during primary response. We may now speculate on its action in the immune ho In this case, an inflammatory reaction is rapidly evoked, and this will res

a raised concentration of antibody in the graft bed. This raised concentra-
n of antibody will take place before leukocytes leave the capillaries. In
e case of leukoses, this vascular response may be all that is required. In
er cases, the antibody must either act synergically or antagonistically with
kocytes. Presumably, it does the former.

It would appear that in the homograft reaction, both a delayed type of
sitivity and circulating antibody are needed, and it is difficult to see how
e could function without the other.

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Discussion of the Paper

Eugene D. Day (*Roswell Park Memorial Institute, Buffalo, N. Y.*): A graf tumor, or any other tissue for that matter, is compatible, by definition, w its host if it survives during the life span of the host. *Innate compatibili* thereby, serves as a measure of certain genetic similarities between two gi inbred strains of mice, *i.e.*, between the strain of mouse to which a tumo indigenous and the strain from which a compatible host is chosen. *Inn incompatibility*, antithetically defined, serves as a measure of certain gen differences. Several strains of mice have now been sorted out into a num of histocompatibility categories on the basis of these definitions. Doctor S has shown that many of these histocompatibility categories stem from all varieties of the *H-2* locus on chromosome *IX* of the mouse. The purpos his research was not primarily to elucidate the *nature* of compatibility, bu provide us with several known combinations of compatible or incompat tumors and hosts, and to evolve from these a variety of valuable, incompati tumor-host combinations differing only with respect to incompatibility.

It follows that any changes that occur in a host subsequent to the tra plantation of an incompatible tumor—changes that otherwise would occur—can be earmarked as manifestations of incompatibility and, if spec enough, as bases of methods for the typing of tumors and hosts according the allelic histocompatibility categories to which they belong. The stra specific immunological manifestations of incompatibility, in the hands Doctor Gorer, have given rise to the field of the immunogenetics of transplan tion. His *Antigen II* and the *D* allele of Doctor Snell's *H-2* locus have bec almost synonymous, and many other allelic varieties of *H-2* have subsequen become identified with their corresponding strain-specific antigens. Now t the technical difficulties of working with red and white cell agglutinins, wh arise in incompatible tumor-host systems, have been surmounted by Doct Gorer and Amos, these cross-reacting antibodies have become important r gents in a rapid method for the assay of the histocompatibility categorie which tumors and their hosts belong.

As for the *nature* of the incompatible graft-host systems, there is a fair nount of substantial evidence that some elusive and specific immunological action is indeed intimately involved, and that compatibility merely measures absence. We must be very careful, however, while searching for the anti- ns and antibodies in such a reaction, not to confuse them with innocuous pes of antitumor antibody, *e.g.*, perhaps with the hemagglutinins, which, though very useful in typing histocompatibility categories, yet may possibly ve little to do with incompatibility per se.

There are four important clues which may lead us to discover more about e nature of incompatibility. The first, which stems from Mitchison's work, ovides evidence that some specific kind of antibody, produced in effective antities only in a lymph node regional to the graft site, can *heighten* the tural incompatibility of a mouse. The second clue, offered by the work of aliss, implicates another type of antibody in *destroying* the natural incom- tibility of a mouse. Mitchison's antibody can *restore* this incompatibility. he third clue, which Billingham and Medawar have supplied, shows that compatibility can be *circumvented* by preventing the formation of antibody the otherwise incompatible host. Mitchison's antibody can also *restore* compatibility in this system.

The fourth clue was presented to us for the first time this evening by Doctor orer. The E.L.4 ascites tumor gives rise in an incompatible host to the ual variety of antibodies, among them the hemagglutinins. In addition, wever, it gives rise to a *tumor-specific* type of antibody which does not cross- act with normal tissues. This antibody, when passively transferred, *heightens* e natural incompatibility in incompatible mice, and *confers* upon compatible ice a transient incompatibility which lasts apparently only as long as the tibody. Only time and further research will reveal whether this is a special se or an example of a more general phenomenon. Whichever proves to be ue, it is certain that our knowledge of the nature of the tumor-host relation- ip has been advanced yet another step toward eventual comprehension.

Doctor Glenn H. Algire (*National Cancer Institute, Bethesda, Md.*)*: I ould like to present in this discussion a method for tissue culture *in vivo* in e peritoneal cavity of mice, and some results from a study of transplantation munity using this method.

Diffusion chambers have been developed[1, 2] in which implanted cells survive d proliferate for long periods of time, although separated from tissues of the st by means of membrane filters. Membrane filters of graded porosity were ed to prevent or to permit the passage of leukocytes and macrophages. either ascites nor severe inflammatory reactions developed in response to the ambers.

The chambers were removed after varying intervals and prepared for histo- gic examination as whole amounts.

These methods were applied to a study of the role of host cells in destruction homografts, using embryonic lung rudiments, epidermal cells, and a mammary adenocarcinoma as test objects.[3] When host cells were excluded, th tissues survived if the hosts were either isologous, homologous, or homolog and previously immunized by tissues of the foreign strain. They did survive in immunized heterologous hosts, however, indicating that antibo could pass through these membranes.

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* Department of Health, Education and Welfare, United States Public Health Service, National Institutes Health, Bethesda, Md.

In similar experiments using filters that permitted the entry of host leu cytes and macrophages, these tissues survived in homologous hosts, but no immunized homologous hosts.

In a third group of experiments, spleen cells (largely lymphocytes) w obtained from mice that were either isologous to the test tissues, homolog or homologous and previously immunized. The spleen cells were washed combined in diffusion chambers with the various test cells as described ab and placed into mice that were isologous with the test cells. The test cells w destroyed only when combined with the immunized homologous spleen cells

These results are in accord with the hypothesis that homografts are destro by "immunized" lymphocytes. No evidence was obtained for the presence antibodies to homografts in the extracellular fluids.

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rt III. Ascites Tumors as Assay Tools

ASCITES TUMORS AS CULTURE MEDIA IN QUANTITATIVE GROWTH STUDIES OF VIRAL AGENTS

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The ascites tumor-virus marriage was a prearranged affair. My wife, Doc- r Irena Koprowska, and I were the matchmakers.[1, 2] The father of the bride we consider the word "ascites" as of feminine gender) was Doctor Albert scher of the Biologic Institute of the Carlsberg Foundation, Copenhagen, enmark. The bride, Ehrlicha, was sent over by plane on June 1, 1950, and August 28 of that year the wedding took place. The West Nile virus (which shall consider as masculine in gender, and not, as the Romans did, neuter) s the groom. In spite of the immigration laws, monogamy did not prevail; e polyandrous Ehrlicha was subsequently wed to several other viruses. The arriages were not very happy ones. On September 23, 1950, an event took ace which hardly anyone could have foreseen. The ascites bride was de- royed by one of the viruses, an exotic individual bearing the name of *Bun- mwera* after the forest in Uganda, Africa, where he was born.[2] During the xt five years, many more ascites brides—listed in TABLE 1—were matched th viruses, some of which were from overseas and some of which were of tive breed.[3-8]

In all, 14 tumors were used: 11 mouse tumors of different histological origin, th different properties as far as propagation in mouse strains is concerned, d 3 rat tumors—Yoshida and 2 hepatomas.[9] The origin of all tumors was ry carefully investigated, and the identity of a given line carried on in this boratory was checked by submitting samples to outside investigators who e authorities in the field, as, for instance, Doctor Klein or the consulting itor of this monograph. The stock supply of several tumors was also oc- sionally "refreshed" by the inclusion of material obtained from other labora- ries where the same lines had been carried in parallel.

The technique usually employed in the experiments consisted of the following eps:

(1) A suspension of free tumor cells was injected intra-abdominally into 12 ice susceptible to a particular tumor line and, three to four days later, these imals were divided into two equal groups. Six mice were inoculated intra- ritoneally (or less often by other routes) with a virus suspension of pre- termined titer. The other six mice received noninfected control material of ouse brain, chick embryo, or tissue culture origin.

(2) Four to five days after inoculation with virus, if no signs of sickness ere noted before, the abdomens of the animals were tapped and ascitic fluid s withdrawn. Aliquots of the fluid were examined in the following ways:) a smear was made and stained for morphological examination; (b) a cell

TABLE 1

TYPE OF TUMOR, SIZE OF INOCULUM AND MOUSE HOST EMPLOYED IN THE STUDY

Original histology	Name of tumor	Modal ploidy	No. of cells in inoculum × 10^6	Mouse strain
Carcinoma	Ehrlich	4n	10–12	Swiss ICR
		2n+	12	
	Krebs 2B	4n	1–5	
Mammary adenocarcinoma	Sarcoma 37*	4n	10–12	
	TA3	4n	10	A
		2n	1–5	A or ICR
Lymphosarcoma	Lymphoma 1‡	4n	50–80	A
	DBA lymphoma	2n	12	DBA or F_1
	6C3HED†	2n	6–10	C3H or F_1
		2n+	10	Swiss ICR
Rhabdomyosarcoma	MC1M†	Sub-4n	1:4 Dil.	C3H

* Changed into sarcoma.

† Originally chemically induced. The other tumors, except lymphoma 1, arose spontaneously in var. mouse colonies.

‡ Radiation induced.

suspension was injected subcutaneously into susceptible mice for bio-ass purposes; and, (c) after centrifugation, the supposedly virus-infected sup natants, free from tumor cells, were titrated in mice (intracerebrally) or in t sue culture.

(3) If this preliminary screening procedure indicated that tumor cells w either damaged or destroyed by the presence of virus, a more quantitative periment was performed. Mice, after being injected with tumor cells a with the viral agent, as above, were tapped daily from the first day of vi injection. Smears of ascitic fluid were examined immediately for the deg of damage inflicted on the malignant cells by virus infection. When a la percentage of cells was found to be damaged, a bio-assay was performed a the virus concentration of the ascitic fluid determined, as above.

In the course of five years of study there were, of course, many deviati from the standard procedures listed. In several instances, determinations virus content were made on the daily aliquots of ascitic fluid, whether or cellular changes were noted. In some cases, mice used were of a strain resist to the lethal action of certain viruses, which made possible the determinat of the oncolytic action of the virus in the originally inoculated host with recourse to bio-assay.

Viruses Which Failed to Multiply in Ascites Tumors

The data on viral agents which either failed to multiply in ascites tumors multiplied at a very low level are summarized in TABLES 2 and 3. It can observed that several viruses gave consistently negative results, regardless the type of ascites tumor used as medium. These were: eastern and weste equine encephalitis, Semliki Forest, herpes, poliomyelitis, and fowlpox. virus has ever been found which will multiply in the Yoshida rat tumor, ev including such highly oncolytic agents for mouse tumors as Mengo, Bunya

TABLE 2
VIRUSES WHICH GENERALLY FAILED TO MULTIPLY IN ASCITES TUMORS
Part 1

	Ehrlich	Krebs 2B	MCIM	Sarc. 37	DBA lymphoma
ngue	X	X	X		X
E	X*	X*	X*	X	X
EE	X	X*	X	X	X
wlpox	X	X	X	X	X
rpes	X	X		X	X
aya	X	X	X		X
liomyelitis	X				X
nliki Forest	X	X			X

* Low level of viral multiplication occasionally observed. Hog cholera, pseudorabies, rabies, and yellow er also failed to multiply in Ehrlich.
X = failed to multiply.

TABLE 3
VIRUSES WHICH GENERALLY FAILED TO MULTIPLY IN ASCITES TUMORS
Part 2

	TA3	Yoshida	Rat hepatoma	6C3HED
ngue	X			X
E	X	X	X	X
EE		X	X	
wlpox	X			X
rpes				X
B			X	X
uping ill		X		
T				X
aya	X			X
nliki Forest	X	X	X	X

X = failed to multiply.

ra, and West Nile viruses, which are not mentioned in the tables. It is ssible that some of the viruses which failed to infect the ascites tumor cells ght indeed multiply if serial passages of the agent were carried out in an cites tumor, either grown *in vivo* or maintained in tissue culture (see below).

Multiplication and Oncolysis

FIGURE 1 indicates the spectrum of viral activity in ascites tumors as reprented by 10 viruses and 10 tumors. All the viruses, with the exception of est Nile, multiplied in six of the 10 tumors: Ehrlich 4*n*, sarcoma 37, MC1M, ebs 2B, Ehrlich 2*n* and TA3 4*n*. Mengo and Bunyamwera viruses were und to grow, in addition, in lymphoma No. 1, and equivocal results were obined as to their multiplication in DBA, 6C3HED, or TA3 2*n* tumors. A rallel between multiplication and oncolytic action could be distinguished only Mengo, Bunyamwera, and West Nile viruses. Multiplication of these three uses was accompanied by oncolytic action. The interval between infection d time of oncolysis was shorter in mice infected with Mengo and Bunyamra than in animals infected with West Nile.

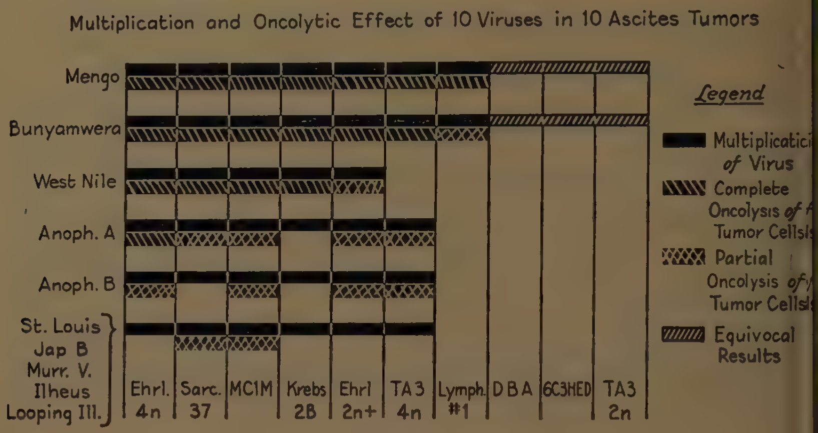
Multiplication and Oncolytic Effect of 10 Viruses in 10 Ascites Tumors

FIGURE 1

No parallel between multiplication and oncolytic action was observed as as the other seven viruses were concerned. Anopheles A virus produced l colysis in five of the six tumors in which it multiplied. Complete oncoly however, was noted only in the Ehrlich 4*n* tumor. Anopheles B virus mu plied in the same six tumors as Anopheles A, but only partial oncolysis of tu cells was observed in four of the six tumors. The same types of tumor which the Anopheles B virus grew also supported the growth of the remain five viruses (the St. Louis-Jap B group), and yet oncolytic action—and t partial—was observed only in sarcoma 37 and MC1M.

These data, of course, relate to viruses propagated in mouse brain, ch embryo, or cultures of normal tissues. If these agents were to be passa serially in ascites tumors, their ability to lyse tumor cells might und changes. It may be stated categorically, however, that no viral agent observed to possess oncolytic properties for a specific tumor if it failed to m tiply in cells of that tumor. Incidentally, Newcastle disease virus, to wh such properties have been attributed,[10] was found by my colleagues A. Flanagan and Doctor R. Love[11] to be devoid of oncolytic power against Ehr ascites cells until it multiplied in such cells.

More Quantitative Aspects of Virus-Induced Oncolysis

TABLE 4 illustrates the influence of the cell concentration in the tumor ino lum on the outcome of virus-induced oncolysis. The West Nile virus and t Ehrlich ascites tumor in PRI mice were chosen for this experiment, since m of this strain are resistant to the lethal effect of the virus.[12] The fate of t inoculated animals, therefore, indicated the success of oncolysis. It can seen that when the inoculum consisted of 16×10^6 cells, only 50 per cen the virus-infected mice survived, but the incubation period was much lo than in the case of the noninfected controls. A decrease in the concentra of tumor cells was accompanied by a more pronounced oncolytic effect of v infection, as evidenced by recovery of infected animals from the malignan

TABLE 4

ATIONSHIP BETWEEN CELL CONCENTRATION OF TUMOR INOCULUM AND PROTECTION THROUGH VIRUS INFECTION

(West Nile virus in Ehrlich ascites*)

ber of cells in culum × 10^6	Tumor mortality and time of death of mice			
	Virus infected		Controls	
	Per cent deaths	Median day of death	Per cent deaths	Median day of death
16	50	29 ± 5.9	100	16 ± 2.3
8	17	13	100	15 ± 3.3
4	17	17	100	22 ± 4.0
2	0	—	83	19 ± 2.3
1	0	—	83	14 ± 0.8

In PRI mice.
Standard error.

TABLE 5

ATIONSHIP BETWEEN CELL CONCENTRATION OF TUMOR INOCULUM AND PROTECTION THROUGH VIRUS INFECTION

(West Nile virus in Krebs 2B ascites*)

nber of cells in oculum × 10^6	Tumor mortality and time of death of mice			
	Virus infected		Controls	
	Per cent deaths	Median day of death	Per cent deaths	Median day of death
8	100	15 ± 3.5	100	7 ± 0.6
4	50	11 ± 6.5	100	9 ± 1.1
2	83	31 ± 7.4	100	10 ± 6.8
1	83	38 ± 7.3	100	11 ± 10.4

In PRI mice.
Standard error.

n some experiments, it was necessary to gauge the effect of viral infection calculating the median day of death rather than by enumerating surviving nals. One such experiment is illustrated in TABLE 5. The Krebs 2B[13] ior is a highly virulent ascites tumor which often kills mice within five to t days after implantation. When tumor inocula consisting of eight, four, , and one million cells were followed by infection with West Nile virus, not re than 50 per cent of the infected animals, at the best, recovered. Yet is-infected mice lived longer than noninfected controls. These data were firmed further by the results of bio-assay: virus-infected cellular suspenns failed to induce subcutaneous tumors upon transplantation.

he concentration of virus in the inoculum may influence the final outcome n experiment. Data summarized in TABLE 6 seem to indicate that neither very lowest nor the very highest dilution of a West Nile virus suspension uced as marked an oncolytic effect as dilutions "in-between." This apent *zone phenomenon* was not infrequently noted in observing the oncolytic ct of other viruses upon other ascites tumors, and it may perhaps reflect a

Table 6

Concentration of Virus in Inoculum and Oncolytic Effect
(West Nile virus in Ehrlich tumor)

No. cells in inoculum × 10^6	Dilution of viral suspension	Mortality ratio of mice*	Median day of death	Day of oncoly
12	1.3	4/7	11 ± 0.95	9
	2.0	2/6	6	9
	2.7	1/6	12	9
	3.4	1/5	12	7
	4.1	4/6	21 ± 11.5	8
	None	6/6	14 ± 3.0	—

* PRI strain.
± Standard error.

Table 7

Multiplication of Bunyamwera Virus and Oncolysis After Inoculation o
Ascites-Bearing Mice by Different Routes

Route of virus inoculation	Experiment	Titer of virus in ascitic fluid	Oncolytic effect days after virus inoculation	Bioass (tumor ra
Intravenous	A	N.T.	5	0/4
	B	6.0	4	0/6
Intra-abdominal	A	N.T.	4	0/4
	B	5.8	4	0/3
Subcutaneous	A	N.T.	10	0/6
	B	No fluid	6	—

blocking of the cells by dead virus particles, which are in highest concentra in the lowest virus dilution.

The route of administration of the virus has some bearing on the final come of an experiment, as shown in TABLE 7, which deals with the effec Bunyamwera virus on the Ehrlich ascites tumor. There was little, if difference between the intravenous and intra-abdominal routes as far as m plication of the virus and time of oncolysis were concerned. There was a sl difference when the virus was injected subcutaneously. In experimen oncolysis was observed 10 days after administration of virus. In experim B, the ascitic fluid disappeared by the sixth day, a confirmation of the mi scopically observed oncolysis. Thus, whatever route was employed, the v always found its way to the ascites cells, multiplied, and induced an oncol effect.

Tissue Culture Studies

In our hands, ascites cells have not been propagated in tissue culture. other words, maintenance of a free-cell suspension in tissue culture did lead to mitosis and appearance of daughter cells. Without undergoing mit the malignant cells, however, remained viable for a time long enough to port the growth of viruses.

When viral agents were introduced into an ascites cell system mainta

TABLE 8

K OF CORRELATION IN SUSCEPTIBILITY TO BUNYAMWERA VIRUS* INFECTION BETWEEN ASCITES TUMORS MAINTAINED IN VIVO AND IN TISSUE CULTURE

Tumor	Multiplication of Virus		
	In Tissue Culture		*In vivo*
	Hours after infection	LD_{50} titer** of virus in mice	
rlich	6	2.5	+
	48	0	
ebs 2	24	0.8	+
	72	0.5	
rcoma 37	24	2.5	+
	72	1.5	
3A lymphoma	24	1.4	−
	72	0	
3 2n+	24	3.5	−
	72	3.5	

* Ten infectious doses used as inoculum.
** Expressed as log to base of 10.

vitro and their growth curve determined, it immediately became apparent
at results obtained in ascites cells grown *in vivo* cannot be applied to the
me system maintained *in vitro*. TABLE 8 summarizes these paradoxical re-
lts. Bunyamwera virus multiplied readily in Ehrlich, Krebs 2, and sarcoma
ascites cells *in vivo*, yet if any multiplication was obtained *in vitro*, it was
obably limited to one cycle. In marked contrast, the diploid TA3 tumor,
ich hardly supports the growth of Bunyamwera virus when implanted in
ce (*v.s.*), has proved an excellent medium for the maintenance of viral
ltiplication *in vitro*. Using this system, the Bunyamwera virus was propa-
ted serially through 80 passages. The resulting strain, however, still lacked
e ability to produce oncolysis in the TA3 diploid tumor in mice.

Mengo virus multiplied well in Ehrlich ascites tumor cells maintained *in
ro*. Although the rate of multiplication of the virus was not as high, as shown
TABLE 9, as in the same cell system grown in the mouse, the *in vitro* data are
rhaps more accurate, since they present results for a system in which one
sue only was available for viral multiplication. The propagation of Mengo
rus in Ehrlich ascites cells *in vitro* has led to very interesting changes in the

TABLE 9

RATE OF MULTIPLICATION OF MENGO VIRUS IN EHRLICH ASCITES MAINTAINED IN VIVO AND IN VITRO

Ascites in	LD_{50} titer* in mice of mengo virus from ascites fluid per virus dilution originally injected					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
ce.	7.5	6.5	6.5	6.5	6.5	<1.0
ssue culture.	6.5	5.3	3.5	4.3	2.5	NT

* Expressed as log to base of 10.

TABLE 10
CHANGES IN PATHOGENICITY OF MENGO VIRUS DURING PROPAGATION IN EHRLICH ASCITES TISSUE CULTURE

Virus passage in ascites tissue culture	Virus titer* in mice		
	Adult		Baby
	I.P.	I.C.	I.C.
0	6.5	6.2	NT
38	1.0	3.5	6.9
60	<Undil.	5.5	NT
68	<Undil.	4.5	>5.5
93	NT	2.5	>5.5

* Expressed as log to base of 10.

properties of the virus, which can be only sketchily described, at present, cause of lack of time.

From data presented in TABLE 10, it may be seen that the original nonada strain is equally infectious for adult mice injected either intraperitoneall intracerebrally. In the course of 38 passages in Ehrlich ascites cells, the enteral invasiveness decreased considerably. Moreover, the virus bec much less pathogenic for adult mice than for baby mice when injected in cerebrally. Further passages of the virus in the same system deprived pathogen of its parenteral invasiveness for mice, and the difference in in cerebral pathogenicity between adult and baby mice remained as wide became even wider. That the increased affinity of the virus for ascites may have been responsible for these changes in its characteristics is sugge by data summarized in TABLE 11. It may be observed that virus at the passage level was one hundred times more infective for the tissue culture tem than for baby mouse brain, which, in turn, was more susceptible than CNS of adult mice.

What Makes a Given Ascites Tumor Susceptible or Resistant To Viral Infection?

Obviously, no definite answer can be given to this question, as the meth employed, so far, are too crude to permit a quantitative analysis of the

TABLE 11
COMPARATIVE SUSCEPTIBILITY OF ASCITES CELLS TO INFECTION WITH MENGO VIRUS* IN VITRO AND IN MICE

Titration in	Infectivity per dilution						
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10
Tissue culture	+	+	+	+	+	+	+
Baby mice	+	+	+	+	+	–	–
Adult mice	+	–	–	–	–	–	–

* Ninety-ninth passage in Ehrlich ascites maintained in tissue culture.

TABLE 12

CHROMOSOME PLOIDY AND SUSCEPTIBILITY TO VIRUS-INDUCED ONCOLYSIS OF MOUSE ASCITES TUMORS

Tumor	Per cent diploid cells	Per cent aneuploid cells	Per cent tetraploid cells	Susceptibility to oncolysis
Krebs 2B	0	92	8	+
Ehrlich-Lettré	0	100	0	+
Ehrlich tetraploid	2	48	50	+
Sarcoma 37	20	52	20	+
MC1M	0	99	0	+
TA3 hypotetraploid	2	94	4	+
TA3 diploid	46	48	6	−
6C3HED	72	21	7	−
DBA lymphoma	49	50	1	−

phenomenon. Micromanipulation techniques, by which a single tumor cell can be isolated and then either placed in a viral suspension or injected directly with a virus, may help to elucidate the problem. It is important to find out whether a given virus does not multiply in an ascites cell because it cannot penetrate the cellular membrane, or because, after it enters the cell, it does not find a suitable substrate for multiplication. Perhaps an even more puzzling problem is presented by the fact that some viruses multiply in the ascites tumor without affecting the viability of the tumor cells.

Data in TABLE 12 resulted from an attempt to correlate the susceptibility of ascites tumors to the oncolytic action of viruses with the chromosome mode of the malignant cells as presented by Hauschka, Levan, and—in one instance—by Kaziwara.[13-16] It appears that cells characterized either by aneuploidy or tetraploidy are, in general, susceptible. In marked contrast, cells which are primarily diploid are resistant to virus infection, and even more resistant to the oncolytic action of the pathogens. It is true that the TA3, $2n$, and DBA lymphoma shown here have an equal percentage of aneuploid and diploid cells. It should be remembered, however, that Levan and Hauschka[13] found that the most frequently encountered cell in the aneuploid class was one with 45 chromosomes and no more. Whether the relationship between the chromosomal ploidy of a tumor and susceptibility to viral oncolysis is a true one remains to be seen. It is possible to speculate that viruses which display a partial oncolytic effect against a polyploid tumor may destroy only aneuploid and tetraploid cells, leaving diploid cells intact. This may be verified by observations of the chromosome modes of cells before and after virus passage in ascites tumors.

Some evidence has recently been observed that chromosome distribution in ascites tumor cells may have something to do with susceptibility to the oncolytic action of a virus. The 6C3HED tumor, which, as can be seen from TABLE 12, is primarily of diploid mode, was recently adapted to Swiss mice by a technique developed by Billingham *et al.*[17] The methods by which the adaptation was accomplished were described last month at the meeting of the American Association for Cancer Research in San Francisco[18] and details will not be presented here. However, FIGURE 2 illustrates the procedure. Fetuses of Swiss

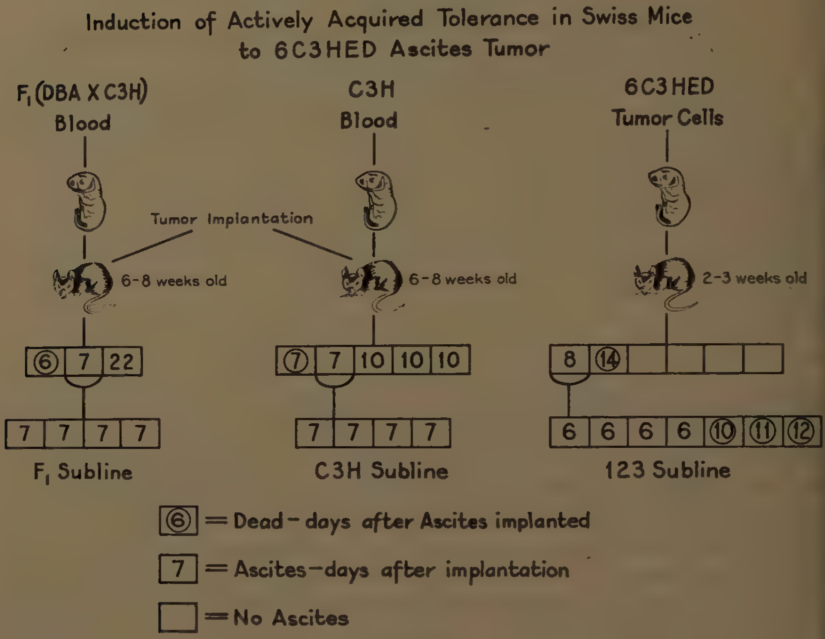

FIGURE 2

mice were injected *in utero* with one of the following: (1) blood of C3H mouse (2) blood of the F_1 generation of C3H × DBA; (3) actual tumor suspension Following either the "conditioning" or direct inoculation of tumor cells, three lines of 6C3HED were established in Swiss mice. When viruses were introduced into these derivative sublines of the tumor, it was quite surprising to find that (TABLE 13), in marked contrast to the behavior of viruses in the origina tumor, all three derivative lines supported the multiplication of Bunyamwer and Mengo viruses, and the malignant cells were destroyed by the viruses.

A preliminary analysis of the chromosome modes of tumors of the three new lines was made by Doctor Hauschka, and data are presented in TABLE 14

TABLE 13
SUSCEPTIBILITY OF THE ORIGINAL AND THREE DERIVATIVE LINES OF 6C3HED TUMOR TO ONCOLYTIC ACTION OF MENGO AND BUNYAMWERA VIRUSES

Tumor line	Virus used	LD_{50} titer* of ascites fluid	Day of oncolysis	Bio-assay (tumor ratio)
Original	Mengo	4.5	—	3/3
	Bunyamwera	2.8	—	6/6
F_1	Mengo	>9.5	2	0/2
	Bunyamwera	4.5	4	0/4
C3H+	Mengo	7.5	2	0/4
	Bunyamwera	4.5	3	1/4
123	Mengo	7.5	2	3/6
	Bunyamwera	5.3	3	0/3

* Expressed as log to base of 10.

TABLE 14
CELL VOLUME AND DISTRIBUTION OF CHROMOSOME COUNTS IN THE ORIGINAL AND DERIVATIVE SUBLINES OF 6C3HED ASCITES

Tumor	Cell volume (cubic μ)	Chromosome number	Number of cells
Original	463	S = 40	26
		2S	1
		4S	0
F_1	610	S = 43–48	20
		2S = 97, 101	5
		4S	0
C3H	493	S = 48–56	14
		2S	10
		4S	1
123	651	S = 46	17
		2S	3
		4S	1

It may be observed that the original tumor, as mentioned before, was characterized by a diploid chromosome mode, whereas the derivative tumors were characterized by polyploidy, with a hyperdiploid mode prevailing. Tumor cell volumes may give another clue as to susceptibility to viral infection and oncolysis, since cells of two of the sublines are markedly larger than those of the original tumor.

Biochemical Studies of the Virus-Ascites Tumor System

The ascites tumor is eminently suitable for biochemical studies, and the infection of freely growing ascites tumor cells with an oncolytic virus provided a splendid opportunity to investigate specific biochemical changes found in the process of viral multiplication and oncolysis.

During the past two years, my colleague, Doctor Colter, has conducted such investigations.[19] The activities of six enzyme systems in the ascites cell; *i.e.* succinoxidase, malic dehydrogenase, aerobic glycolysis, anaerobic glycolysis, acid phosphatase, and beta-glycuronidase were found to be unaffected by Bunyamwera virus infection. Several enzymes were found to be totally absent from normal and infected Ehrlich cells: alkaline phosphatase, choline oxidase, and desoxyribonuclease. The only enzyme system in the Ehrlich ascites cell found to date to be affected by the multiplication of Bunyamwera virus is arginase. As seen in FIGURE 3, infection with the virus stimulated arginase activity, maximum values being observed 24 to 30 hours after inoculation. Thereafter, the arginase activity declined, reaching normal levels 48 hours after infection. The values obtained at 72 hours were significantly lower than in noninfected cells.

Changes observed in arginase activity in the infected cells were accompanied by alterations in arginine values. FIGURE 4 indicates the results obtained in several experiments, and shows that arginine was the only amino acid of the Ehrlich ascites tumor cell affected by Bunyamwera virus infection. The arginine content of the cell was low 24 hours after infection, at the time maximum values were obtained for arginase. It returned almost to normal at 48 hours,

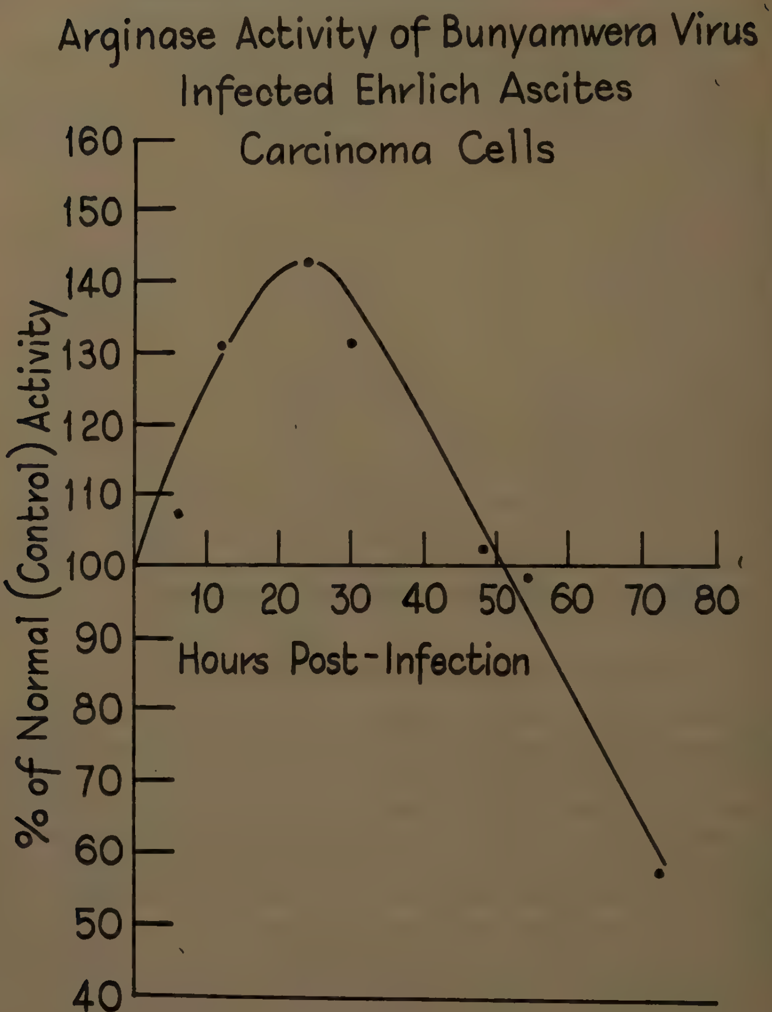

FIGURE 3

and rose above normal at 72 hours. The last stage coincided with the drop i arginase activity.

In FIGURE 5, data are summarized on RNA-DNA ratios of normal and Bunyamwera virus infected Ehrlich cells. Along with the progress of infectio there was a concomitant decrease in the RNA:DNA ratio. This change wa probably the result of increased synthesis of DNA, although the number c determinations was too small to permit definite conclusions.

Electronmicroscopic Studies

Several years ago, Doctor Brown, Doctor Love, and the author became interested in tracing, by means of the electron microscope, the intracellula

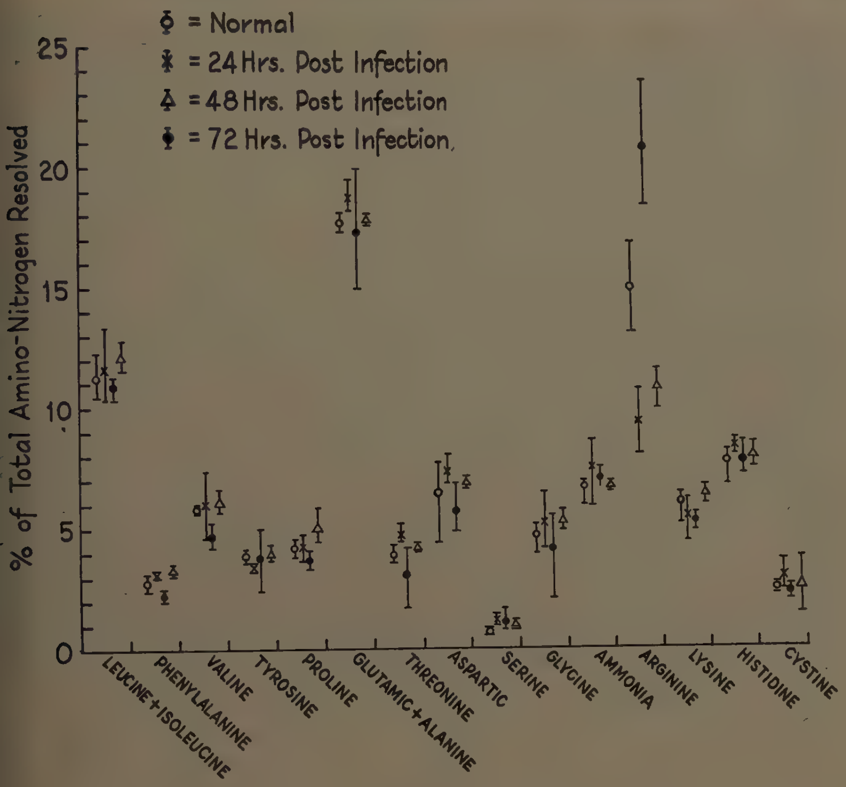

FIGURE 4

pathway of multiplication of an oncolytic virus, and its ultimate cellular structure.

A study was set up in collaboration with the Department of Microbiology, College of Physicians and Surgeons, Columbia University, with Doctors Friedlaender and Moore. Ascites cells infected with Bunyamwera virus were the first electronmicroscopic target. However, minute investigations of the system failed to reveal particles which even remotely resembled a virus. This came as a surprise, since Smithburn and Bugher had estimated the size of Bunyamwera virus, according to the Elford scale, to be 70 to 105 mu,[20] large enough to be seen under the electron microscope. Further investigations of the same virus-cell system were also fruitless. Two questions then arose: Is it possible to see a virus in the ascites cell? Or, are there qualities of the Bunyamwera virus which make it invisible? My colleague, Doctor Brown, attempted to elucidate the second point. His observations, based on the ultracentrifugation of Bunyamwera virus, indicated a particle size of 14 to 22 mu, definitely smaller than had been originally reported. It was not surprising, therefore, that the virus could not be visualized in the ascites cell.

As it seemed that Mengo and West Nile viruses might also be too small for electronmicroscopic work, the next best choice among the oncolytic viruses

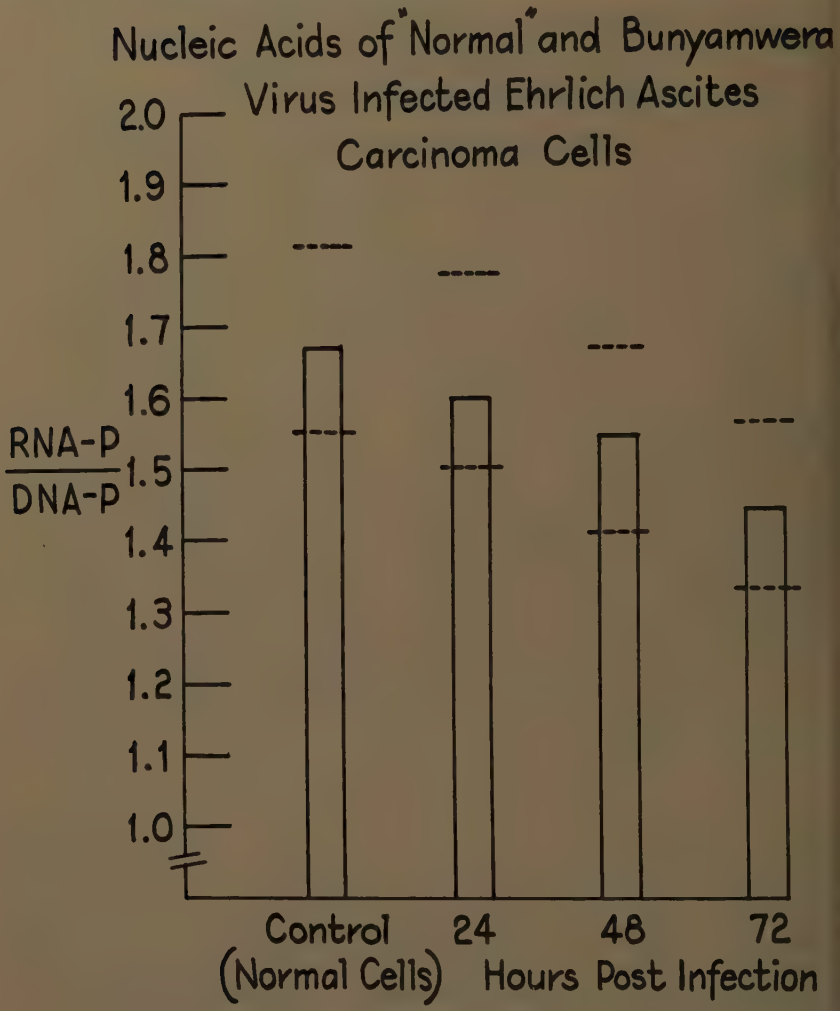

FIGURE 5

was Anopheles A. Investigations of this virus—about which very little i known—in Ehrlich ascites cells were undertaken by Doctor Dan Moore an others. Results were rather striking,[21-23] and will be illustrated in the nex few plates. FIGURE 6 shows an electron micrograph of a normal, noninfecte Ehrlich ascites tumor at 11,500 magnification. There is no need for a detaile description, since this was covered yesterday by Cecily Selby. Attentio should be called to the highly osmophilic aggregates which are present in th cytoplasm and have been named L-bodies[22] in order to differentiate these struc tures from other lipoidal components of the cells.

After the infection of the Ehrlich ascites cell with Anopheles A virus, interest

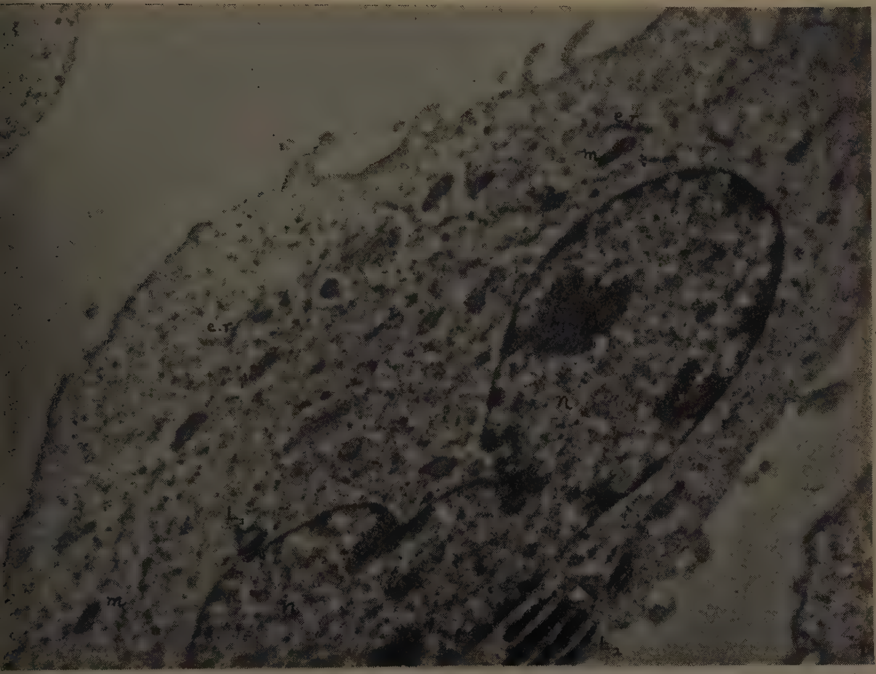

FIGURE 6. Noninfected ascites cell. *n* = nucleus; $L_1 + L_2$ = L-bodies; *m* = mitochondria; and *er* = endoplasmic reticulum. × 11,500.

ing changes were observed within the infected cell. On FIGURE 7 there can be seen an aggregation of particles, which probably represents the virus, in the cytoplasm near the indentation of the nucleus (arrow toward *N*). There is a marked increase in the size as well as in the number of the previously-mentioned L-bodies (*L*). Numerous vacuoles (*v*) are present in the cytoplasm, some in the vicinity of necrotic regions (*VINR*) which are characteristic of the infected cell and increase in number as infection progresses.

Particles which are believed to be Anopheles A virus appear in high magnification (×86,000 to 98,000) on FIGURE 8. The upper left figure shows a pair of particles embedded in an envelopelike formation, and in the upper right figure can be seen two particles sectioned at a different level. The large micrograph shows a cluster of particles near a vacuolelike formation. The virus appears to have a transparent core within a compound electron-opaque shell, and measures 50 to 65 mu, with a probable value of 56 mu. More detailed investigations[23] of the infectious process indicate that the endoplasmic reticulum, which is believed to be an interconnecting canalicular structure, is intimately associated with the entrance, spread, and multiplication of the virus within the cell.

Thus, for the first time, a virus of almost unknown properties has been identified under the electron microscope in its proper milieu: a living cell. The confirmation of its size obtained by my colleague, Doctor Brown, through the study of physical properties of the virus, and the demonstration of similar

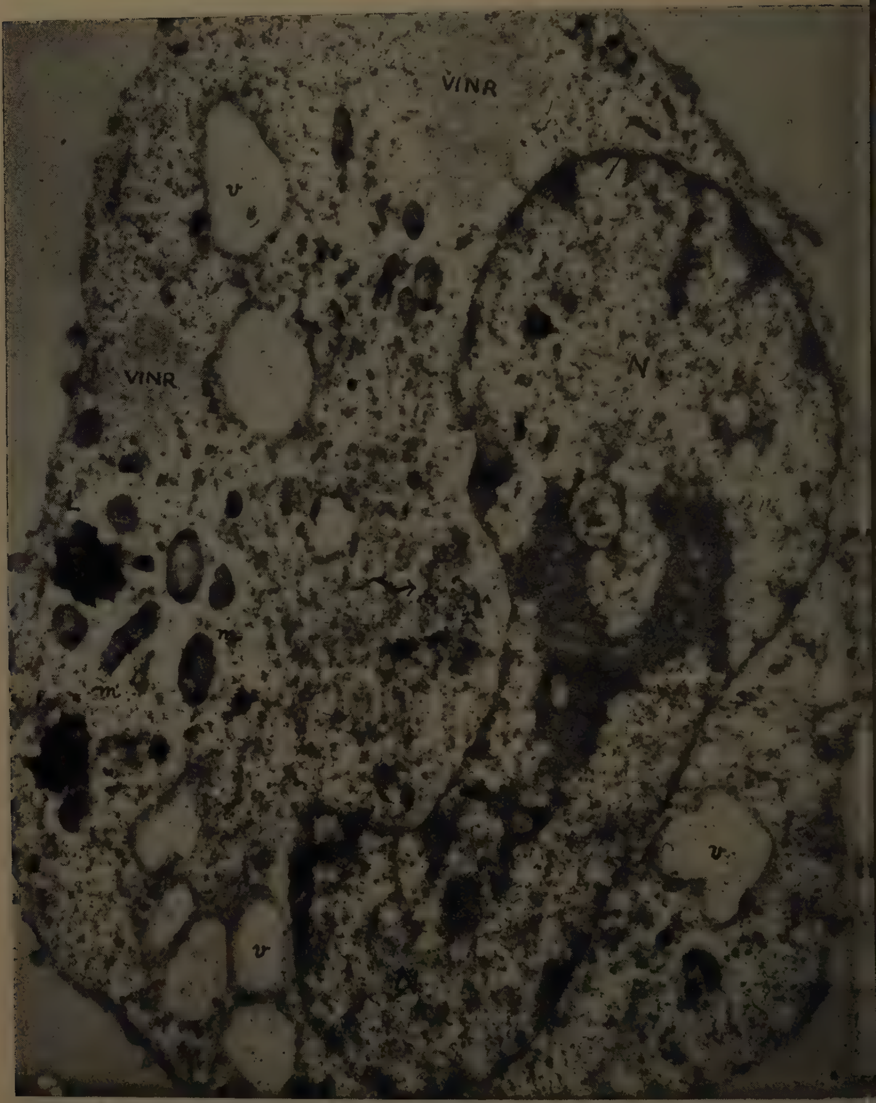

FIGURE 7. Ascites cell infected with Anopheles A virus five days previously. *n* = nucleus; *m* = mitochondria; *L* = L-bodies; *v* = vacuoles; and *VINR* = virus-induced necrotic region. × 9,400.

particles on infected chorio-allantoic membranes make the identification mor certain.

The present, as well as other investigations summarized before, indicate tha the ascites tumor-virus system has become one of the most useful tools in th quantitative evaluation of the relationship between viruses and cells. In addition, the oncolytic phenomenon may one day give us a clue to the mechanism of the selective biological destruction of a malignant cell.

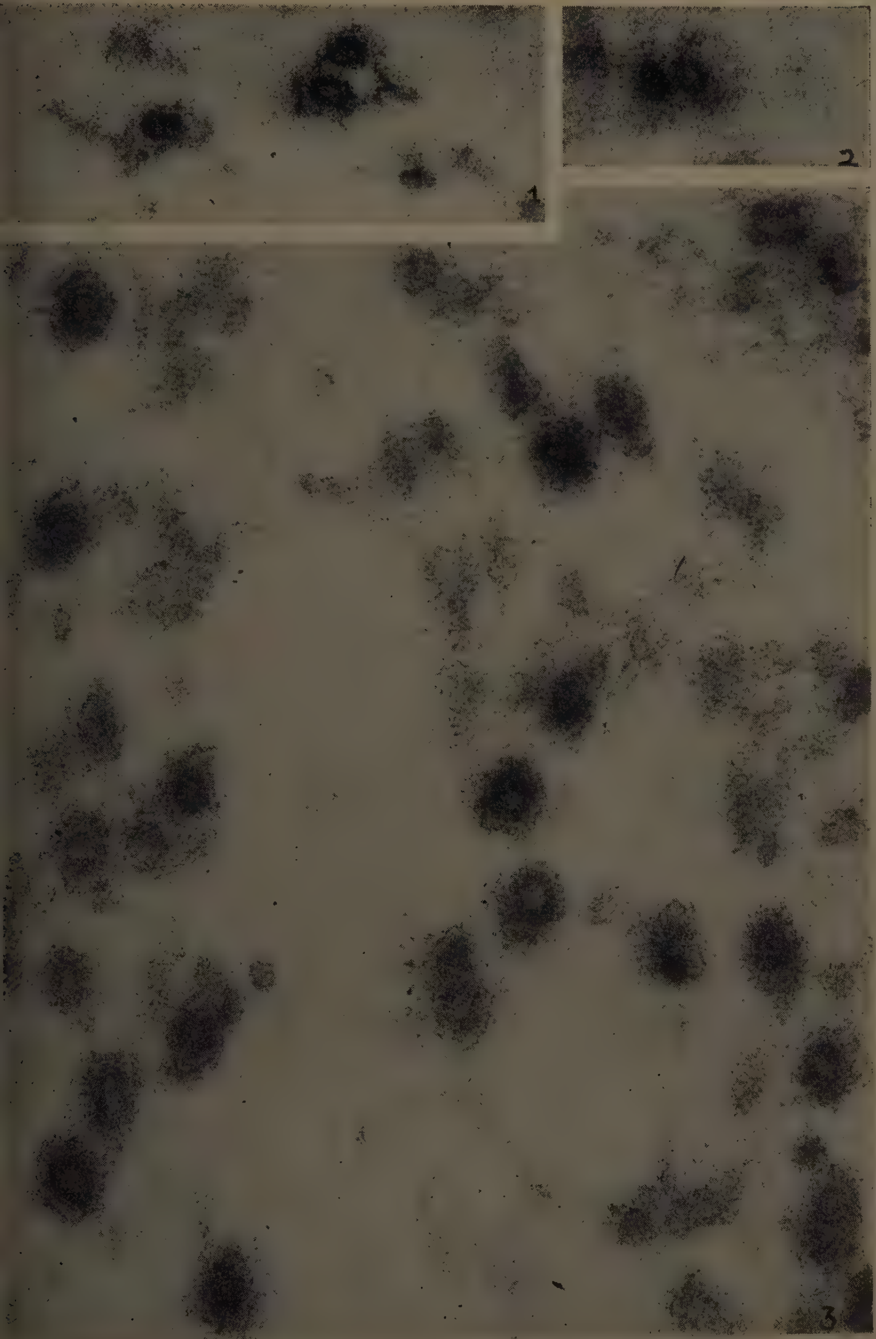

FIGURE 8. A cluster of virus particles in an infected ascites cell. × 86,000 to × 98,000.

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Discussion of the Paper

Doctor Robert Love (*Lederle Laboratories, Pearl River, N. Y.*): Docto Koprowski has given such an excellent—if somewhat gruesome—descriptio of the events which followed the ascites-virus wedding that I hesitate to delv deeper into the affairs of the family. My colleague, Doctor E. V. Orsi, and have been particularly interested in the troubles that have arisen betwee Madame Ehrlich and four of her virus husbands: Messrs. Bunyamwera Mengo, West Nile, and Anopheles A.

A description of the effects of Bunyamwera virus infection on the Ehrlic ascites tumor cell and of the methods employed to study the cellular change has been published.[1, 2, 3] Some of the cytological mechanisms involved i the destruction of the Ehrlich tumor cell by infection with the other three viruses are summarized in TABLE 1. The earliest change in each case is observe in the interphase tumor cell. There is margination of the chromatin an clumping of the parachromatin to form prominent parachromatin bodies. Th nucleoli become shrunken and are displaced peripherally. The inability o these cells to enter the mitotic cycle is clear, since their appearance coincide with a fall in the percentage of mitotic figures in the infected fluid. Karyorrhexis is completed in the cells infected with West Nile and Anopheles A viruse by a process of multiple nuclear budding. The nuclear fragments are surrounded by a chromatin membrane and frequently contain nucleolar remnants or parachromatin bodies. Karyorrhexis proceeds in a similar fashio in the Mengo-infected cells, except that nuclear and nucleolar shrinkage i more striking and the parachromatin bodies are larger. The later stages o nuclear fragmentation are accompanied by disintegration of the cell.

In a sense, these nuclear changes cannot be regarded as the specific results c

TABLE 1

MECHANISM OF DESTRUCTION OF THE INTERMITOTIC EHRLICH ASCITES TUMOR CELL BY INFECTION WITH MENGO, WEST NILE, AND ANOPHELES A VIRUSES

Site of change	Nature of change
Chromatin	Margination; failure to enter mitotic cycle; karyorrhexis.
Nucleolus	Shrinkage and peripheral displacement.
Parachromatin	Clumping to form parachromatin bodies.
Neutral lipid globules	Hypertrophy and hyperplasia.
Lipid and glycoprotein of lipochondria	
Mitochondria	Displacement by increased lipochondria.

virus infection, since any or all of them may be observed in a rare cell of an uninfected tumor. The proportion of uninfected tumor cells showing these changes never exceeds one per cent whereas, after virus infection, as many as 80 to 100 per cent of the tumor cells are affected.

Contemporaneous with the nuclear changes, the cytoplasmic particulates of the infected tumor cells undergo striking alterations which have never been observed in uninfected cells. At least three different types of sudanophilic particles have been demonstrated in the Ehrlich ascites tumor cell.[1, 4] The largest consist of large droplets of neutral fat which, for want of a better term, have been called neutral lipid globules. The smallest sudanophilic organelles are the mitochondria. A third type, which consists of glycolipid and glycoprotein, is an aggregation of ill-defined granules mainly in the cell center or nuclear hof area of the cytoplasm. Since some of these particles appear to stain supravitally with neutral red, they presumably correspond to the lipochondria as defined by Ries[5] and the Oxford school.[6] During the early stages of nuclear disorganization in the virus-infected tumor cells, there is a remarkable hypertrophy and hyperplasia of the neutral lipid globules and of the lipochondria. An increase in the number and size of the lipochrondria can be demonstrated in fixed cells after infection with any of the three viruses. This effect is most conspicuous in the cells infected with West Nile virus, in which there is also a definite increase in the number and size of the supravitally stainable neutral red particles.

Although no morphological changes can be demonstrated in the mitochondria of the infected cells by the toluidine blue staining method,[3] they are displaced to the periphery of the cytoplasm by the accumulation of enlarged lipochondria in the nuclear hof. Within 24 hours after inoculation of Mengo virus, the mitochondria no longer stain supravitally with Janus green.

The enzymes of the succinoxidase system which are demonstrated by Rosa and Velardo's neotetrazolium method[7] are impaired by infection with any of the three viruses. The enzyme activity is most severely affected in Mengo-nfected tumor cells where, 24 hours after infection, practically no color is produced after two hours' incubation in the substrate.

Two or more days after infection of the tumor with Anopheles A virus, one or several clear structureless ovoid areas (1 to 8μ long) are observed in the cytoplasm of many living neoplastic cells examined by phase microscopy. In the later stages of infection, they are present in practically every tumor cell. Such areas are not seen in the nuclear hof, and they appear to correspond in every respect to acidophilic cytoplasmic "inclusions" which cann be readily stained by Barrett's method.[8] These inclusions contain tyrosine tryptophane or histidine, but no demonstrable lipid, nucleic acids, succinic dehydrogenase, phosphatases, or PAS positive material. Their size and distribution strongly suggest that they are the "virus-induced necrotic regions" described by Friedlaender *et al.* in electron micrographs of similar material.

The changes produced in the Ehrlich ascites tumor by infection with Mengo West Nile, and Anopheles A viruses resemble those which have been described after the infection of tumors with Bunyamwera and St. Louis encephalitis viruses.[1, 2] Hypertrophy and hyperplasia of the lipochondria have been a common feature of all the virus-infected tumor cells that we have studied, and have not been observed in uninfected degenerating tumor cells.[2] In tumor cells of the RPL-12 lymphoma infected with St. Louis encephalitis virus, lipochondrial hypertrophy is accompanied by metaphase arrest. While the teleological significance of the changes in the lipochondria is not clear, their development would appear to be the result of virus infection, and their presence is accompanied by a disturbance of nuclear function which eventually results in regression or oncolysis.

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Doctor M. Michael Sigel (*School of Medicine, University of Miami, Cora Gables, Fla.*): Doctor Koprowski has given a brilliant summation of the extensive studies conducted by himself and his associates on the intimate relations between ascites tumor and a large variety of viruses.

My brief comments concern a system in which the relations are not quite a intimate. To be sure, a close association of virus and tumor appeared to b necessary in this system, but the quality of this association lacked the intimacy of Doctor Koprowski's unions. The meningopneumonitis virus caused th inhibition of Krebs-2 carcinoma without producing observable damage to th tumor cells. The virus did not appear to multiply in these cells. Complete suppression of tumor required that viruses be introduced almost simultaneously with the tumor cells. A delay of one hour led to the formation of tumor in about one half of the animals. This phenomenon was reciprocal in that, unde certain conditions, growing tumor interfered with the multiplication of virus Our findings suggest that, in this system, one may be dealing with a competition between virus and tumor for a metabolite necessary for the growth of both This type of interaction represents a departure from the virus-cell relationship where viruses infect tumor cells, multiply in them and, thereby, cause thei destruction.

AUTORADIOGRAPH STUDIES WITH MOUSE EHRLICH ASCITES TUMOR

By Shirley Hornsey and Alma Howard*
M.R.C. Experimental Radiopathology Research Unit, Hammersmith Hospital, London, England

The autoradiograph method permits a study of the incorporation of a labeled precursor into deoxyribonucleic acid (DNA). It has been shown by means of autoradiographs that, in some plant cells (Howard and Pelc, 1951), and in human bone marrow cells in culture (Lajtha, Oliver, and Ellis, 1954), synthesis of DNA occurs during only a part of the cell cycle. If this is true also of tumor cells, a study of the labeling of DNA in individual cells may afford a means of measuring the length of the mitotic cycle in such cells.

This paper reports an attempt to estimate the duration of the mitotic cycle in the Ehrlich ascites tumor at five days' growth, and some conclusions concerning the time in the cycle during which [8-^{14}C] adenine is incorporated into DNA. If ribonucleic acid (RNA) is removed from the tumor cells by the action of ribonuclease, the activity remaining in the cells can be shown to be present in compounds that are removed by deoxyribonuclease.

Material and Methods

Ehrlich ascites carcinoma of the tetraploid line (Hauschka and Levan, 1951) was obtained in October, 1952, through the kindness of Doctor G. Klein, Stockholm, Sweden, and subsequently maintained in stock mice by weekly transfer of approximately 10^7 cells.

[8-^{14}C] Adenine hydrochloride (specific activity 1 mc per m. mole) was obtained from the Radiochemical Centre, Amersham, Bucks, England. It was dissolved in water and injected intraperitoneally (I.P.) in volumes of 0.2 to 0.5 ml. It is referred to simply as adenine.

Successive samples of tumor cells were obtained from individual mice by withdrawing a small volume of ascitic fluid with a syringe. Different cytological methods were used in different experiments and are described below. Autoradiographs were prepared by the stripping film technique (Doniach and Pelc, 1950). Film was obtained from Kodak, Ltd., Harrow-Wealdstone, Middlesex.

Experiment No. 1. Rate of loss of ^{14}C from ascitic fluid after adenine injection. Adenine (2μC) was injected I.P. into each of five C_{57} Black female mice bearing five-day-old tumors; three to five successive samples of about 0.1 ml. of the ascitic fluid were taken from each mouse at intervals from three minutes to five days after the injection. Each sample was put into a hematocrit tube that already contained approximately 1 international unit of heparin in a minute drop of solution, to prevent clotting. The cells were spun down, and 0.03 to 0.06 ml. of supernatant was removed to weighed planchettes lined with lens tissue. The samples were dried and assayed for ^{14}C with a thinwindow counter. The results are expressed as counts per minute at infinite thickness for a sample 2 cm.^{2} in area.

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Experiment No. 2. Identification of labeled compounds in cells. A C$_5$ Black × C$_3$H F$_1$ female mouse, 7 to 10 weeks old, was injected I.P. with 0.15 ml. fresh ascitic fluid containing about 12 × 10^6 tumor cells. Five days later, it was injected I.P. with 20 μc. adenine and killed four hours later.

The ascitic fluid was mixed with approximately five volumes of 45 per cent acetic acid. The cells were spun down and smeared on slides and dried in air Smears were washed in running tap water and divided into seven groups for further treatment: (*a*) water washing only; (*b*) incubation for 40 minutes at 37° C. in 0.02 M. veronal buffer, at pH 7, containing 0.05 mg. crystalline ribonuclease per ml.; (*c*) incubation for 40 min. at 37°C. in 0.02 M. veronal buffer plus 0.02 M. hydroxylamine, at pH 7, containing 0.05 mg. crystalline deoxyribonuclease per ml.; (*d*) treatment *c* followed by treatment *b*; (*e*) treatment *c* followed by a further 40 minutes incubation in buffer without ribonuclease (*f*) extraction with 1 N HCl at 60° C. for six minutes; (*g*) treatment *f* followed by Feulgen staining. The enzymes were obtained from Worthington Biochemicals Ltd., Freehold, N. J. After treatment, the smears were washed thoroughly in distilled water and coated with stripping film emulsion. Slides used for grain counting were exposed for 13 days (*a*) or 38 days (*b* to *g*).

Experiment No. 3. Labeling of DNA at various times after adenine injection Three tumor-bearing mice similar in all respects to the one used in experiment No. 2 were injected I.P. with 18 to 19 μc. of adenine. Injection of the three mice was made from the same adenine solution at the same time. Samples of ascitic fluid were withdrawn at intervals. Mouse No. 1 was sampled at 1 hour, 1.5 hours, 2 hours, and killed at 4 hours. Mouse No. 2 was sampled at 30 minutes, 1 hour, 4 hours, 6 hours, 9 hours, and killed at 12 hours. Mouse No. 3 was sampled at 4 hours, 12 hours, 15 hours, 26 hours, and 35 hours. At each sampling, three smears were made of fresh ascitic fluid. These smears were dried in air and fixed in 90 per cent ethanol. After killing, three smears were made in the same way. The remaining ascitic fluid was then washed out and fixed in bulk as in experiment No. 2. After washing with water, all smears were incubated with ribonuclease as in *b*, experiment No. 2, and autoradiographed. All the emulsions on slides used for counting photographic grains in experiment No. 3 were exposed for 34 days.

Method of observation of autoradiographs. The number of photographic grains above background, developed in the area of autoradiograph film above a cell is proportional to the amount of radioactive material present in the cell. Observations were made with a ×95 phase-contrast lens; phase-constrast was used to observe the cells and, by removing the phase plate, the photographic grains could be counted (FIGURE 1). All grains were counted that lay at any level in the film within the area covered by the cell. Grains above 100 to 21 cells in interphase, or 25 to 50 cells in metaphase or telophase were counted for each sample. In experiment No. 2, backgrounds were low (3 to 4 grains/100 μ^2) on all slides and no correction has been made for them. In experiment No 3, backgrounds were high due to the unforseen presence of gamma-ray emitters in the building in which the slides were exposed. Therefore the mean diameter

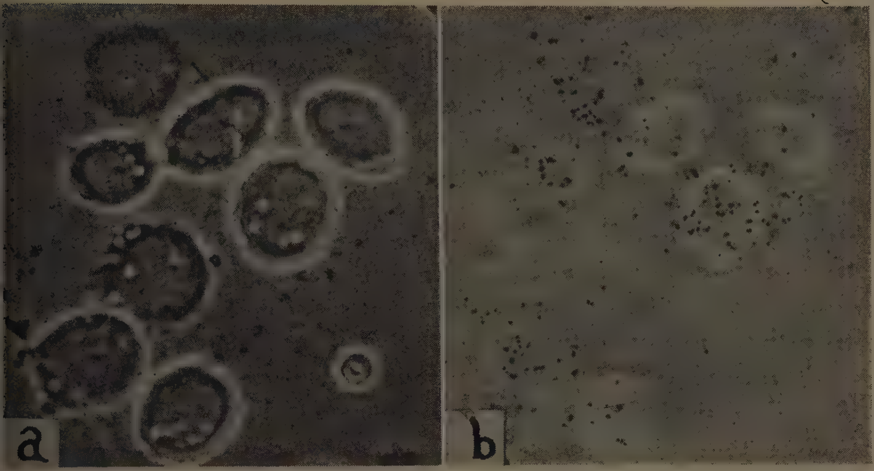

FIGURE 1. Autoradiograph of ribonuclease-extracted smear of Ehrlich ascites tumor. Sample taken six hours after intraperitoneal injection of 20 μc. [8-^{14}C] adenine. (a) Phase contrast, showing cells. (b) Same field, phase plate removed, showing developed photographic grains in the film above the cells. × 930.

of each cell was measured with a micrometer eyepiece at the time of making the grain count, the mean background per unit area was measured, and a correction was made for the grain count of each cell according to its area.

Results

(1) *Activity of ascitic fluid after injection of adenine* (*experiment No. 1*). FIGURE 2 shows the drop in radioactivity of cell-free ascitic fluid with increasing time after injection of 2 μc adenine. The activity falls from nearly 4000 counts per minute at three minutes to about one tenth of this value at one and one-half to two hours after injection of adenine. After about three hours the level decreases slowly.

(2) *Identification of labeled compounds in cells fixed four hours after adenine injection* (*experiment No. 2*). FIGURE 3 gives histograms showing the percentage of cells having various numbers of grains after different extraction procedures. Except in the sample illustrated in *a*, (water washing only), autoradiographs were exposed for 38 days. After treatment *a*, labeling was so heavy as to make grain counting over cells impracticable in the slide exposed for 38 days, and the sample illustrated in FIGURE 3A is from a slide exposed for 13 days only. It is clear that the degree of labeling in this slide, extracted with water only, varies widely between cells and, if a factor of three for exposure time is introduced, that it is considerably greater than in any of the other samples.

When slides are treated with ribonuclease (*b*) a similarly large variation in degree of labeling is apparent between different cells. Treatment with deoxyribonuclease (*c*) gives a much smaller spread of values. The difference between *c* and *d* (extracted with both enzymes) is presumably due to the presence in *c* of cells containing labeled ribonucleic acid (RNA). This conclusion is confirmed by the similarity of distributions found after treatments *c* and *e*.

This labeled RNA, however, must be only a small fraction of that present before fixation, since both the fixative and the buffer in which the slides were incubated with DNase would be expected to remove much RNA.

Hydrolysis in 1 N HCl for six minutes at 60° C. results in a distribution o grain numbers (*f*) very similar to that found after treatment with DNase plus RNase. Such hydrolysis, which is followed by a good Feulgen reaction is known to remove the purine bases from DNA and RNA (Davidson, 1952) Lajtha (1954), however, has found, by the autoradiographic technique, that such hydrolysis did not remove the radioactivity of DNA in human bone-marrow cells cultured in the presence of [8-^{14}C] adenine. Lajtha used methano as fixative: therefore we have examined slides fixed in ethanol as in experiment No. 3 (see methods) and hydrolysed as above. These slides showed no labele cells, indicating a difference in purine solubility due either to a different effec of methanol and ethanol used as a fixative, or to a difference between the tis-sues.

No comprehensive extraction experiment was carried out on smears fixe in ethanol, such as those used in the grain counts reported in experiment No. 3 However, a slide from experiment No. 3 which was fixed in 45 per cent aceti acid (and thus comparable with experiment No. 2) and extracted with ribo-nuclease, was grain-counted and compared with a similarly extracted slide made from the same tumor sample, which had been fixed in 90 per cent ethanol The histograms obtained from the two slides were very similar in degree o labeling and number of labeled cells (FIGURE 4A). Very similar distribution of grain number were also obtained when a sample taken four hours afte

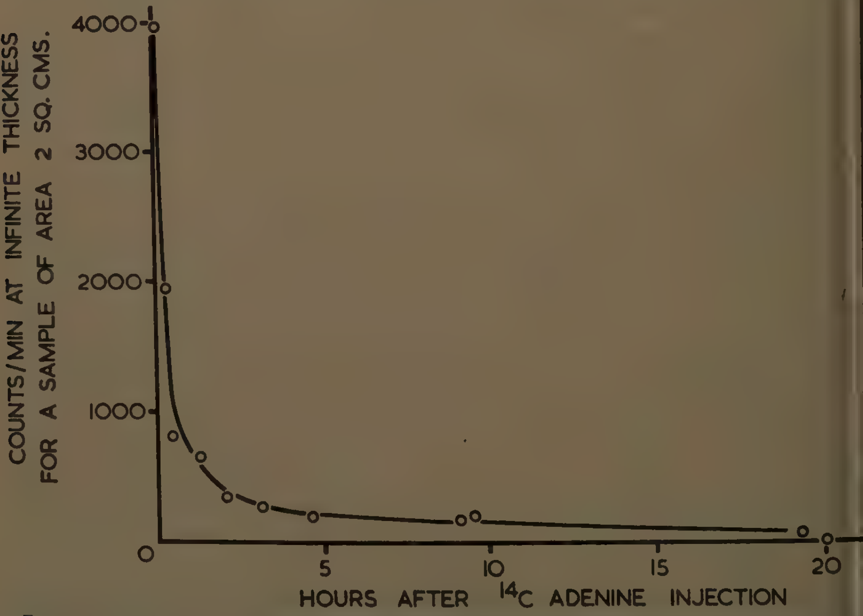

FIGURE 2. Total activity of cell-free ascitic fluid after intraperitoneal injection of 2 μc. [8-^{14}c] adenine (ex periment No. 1).

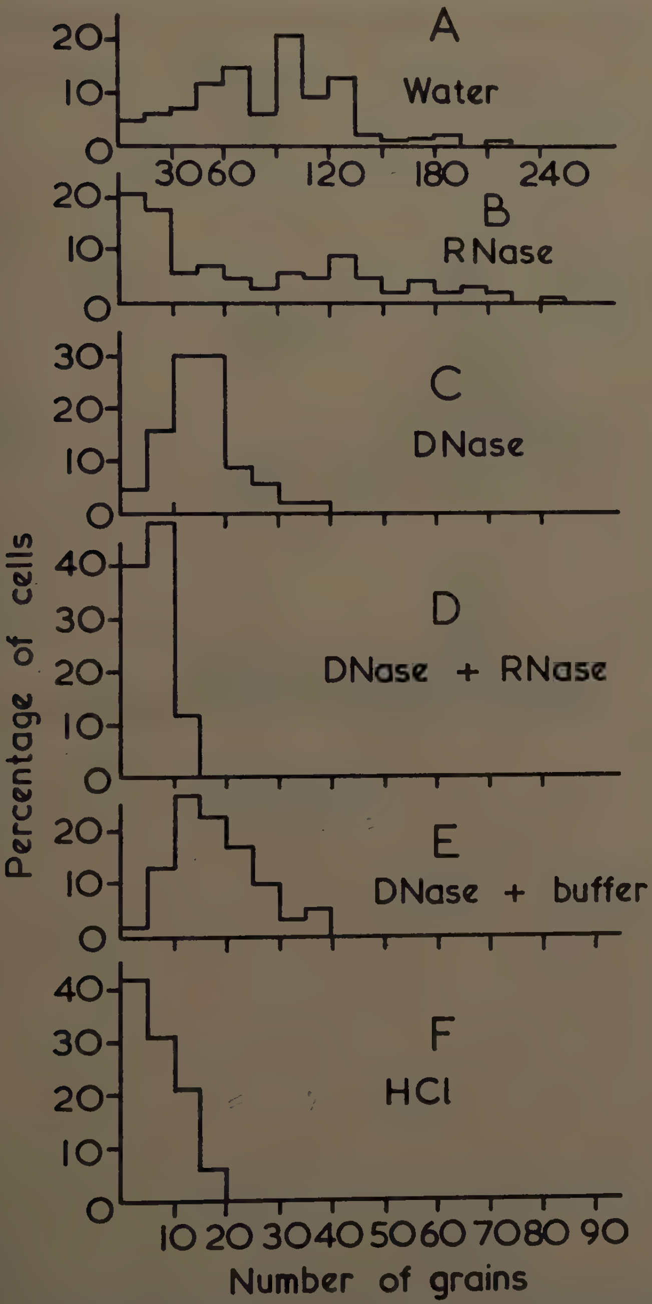

FIGURE 3. Distribution of interphase cells with regard to grain number in smears after different extraction procedures (experiment No. 2). One hundred cells in each sample. No correction for background.

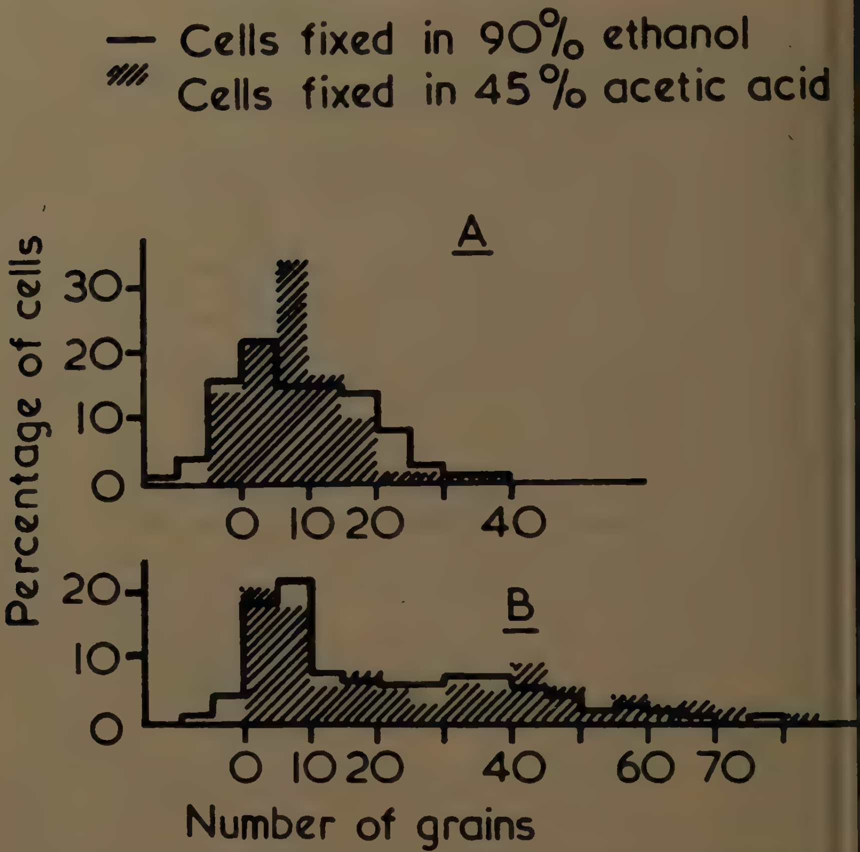

FIGURE 4. Comparison of activity in cells after ribonuclease extraction following two methods of fixatio (a) Two-hour sample, mouse No. 2, experiment No. 3. (b) Four-hour samples 2 and 3. Open: cells fixed in per cent ethanol. Hatched: cells fixed in 45 per cent acetic acid.

adenine injection in experiment No. 3 was compared with the four-hour ribo nuclease-extracted slide of experiment No. 2 (FIGURE 4B). These observation are taken to justify the conclusion that slides of experiment No. 3, fixed in 9 per cent ethanol and extracted with ribonuclease, show autoradiographs du largely to labeled DNA. This conclusion is supported by the fact that area of cytoplasm seen in interphase cells do not show autoradiograph.

(3) *Labeling of DNA at different times after adenine injection (experimen No. 3).* FIGURE 5 gives histograms showing the number of cells in interphase metaphase, and telophase having various grain numbers (corrected for back ground) at different times after adenine injection. The results obtained o the three mice used are shown separately. One of the samples taken (fou hours, mouse No. 2) could not be counted because of the presence in smears c large numbers of blood cells and few tumor cells. This is ascribed to fault withdrawal of fluid.

The samples of interphase cells for which grain counts are shown in FIGUR

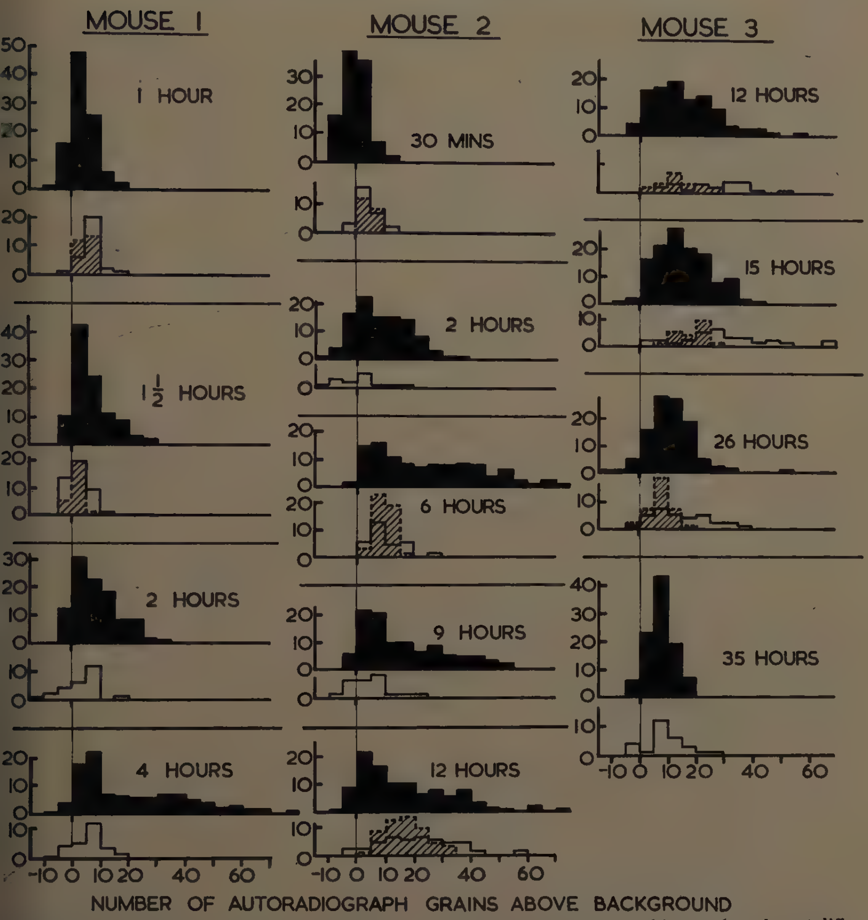

FIGURE 5. Distributions of cells with regard to grain number above background in samples taken at differ- nt times after intraperitoneal injection of adenine. Solid: interphase cells in percentages. Open: metaphases. haded: telophases. Actual numbers of cells counted. For telophases, grain numbers above each daughter ucleus are recorded separately.

5 contain from 100 to 212 cells at each time point. The sampling error is herefore 10 per cent or less.

Interphases. If unlabeled cells were to be classified according to their num- er of grains above background, a symmetrical distribution with a mean of ero would be expected. At 30 minutes after injection (FIGURE 5, mouse 2), his type of distribution, in fact, was observed, and we conclude that there was o labeling of DNA in interphase cells at 30 minutes. At all later times, the istributions of cells with regard to grain number are shifted to the positive ide of zero, though some cells remain on the negative side. Since unlabeled ells would be symmetrically distributed, we have estimated the percentage of abeled cells in the whole sample as the percentage above zero less the percent- ge below zero. These values for each sample are given in TABLE 1 and FIG-

TABLE 1

Time	Mouse No.	Per cent labeled interphase cells	Average No. of grains above background per interphase cell
30 min.	2	0	−1.0
1 hr.	1	66	3.3
1½ hr.	1	80	6.2
2 hr.	1	76	7.5
2 hr.	2	58	8.0
4 hr.	1	90	20.1
6 hr.	2	98	23.8
9 hr.	2	89	15.6
12 hr.	2	81	15.2
12 hr.	3	92	15.3
15 hr.	3	94	18.2
26 hr.	3	86	9.8
35 hr.	3	88	6.8

URE 6, which show also the average number of grains above background pe cell.

Metaphases and telophases. The distribution of metaphase cells according t grain number above background is rather constant at a low level up to nin hours and does not show the increasing degree of labeling that is evident fo interphase cells during this period. At 12 hours, metaphase cells show an abrup increase in degree of labeling. This is indicated by the range of their distribu tion (FIGURE 5) and by their average grain number (FIGURE 6). The degre of labeling of metaphase cells at 12 hours is similar to that of interphase cell at 4 to 6 hours.

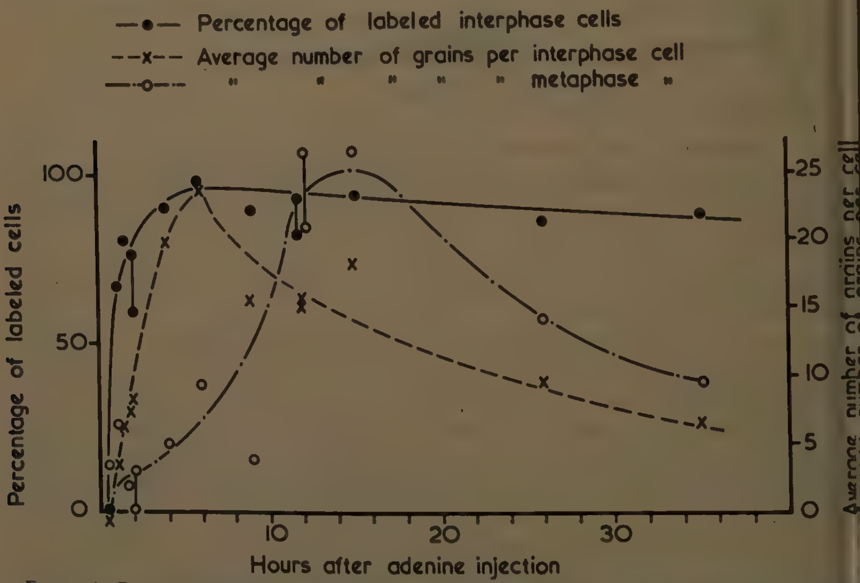

FIGURE 6. Percentage of labeled interphase cells and average number of grains above background over inte phase and metaphase cells at different times after intraperitoneal injection of adenine.

It will be noted that no sample shows a symmetrical distribution of metaphases and telophases about zero. They all show the majority of cells having a positive grain number above background, indicating that at no time do we observe an entirely unlabeled population of dividing cells. The degree of abeling in dividing cells from 1.5 to 9 hours is, however, slight compared with hat of interphase cells at the same times. The marked increase in degree of abeling of divisions at 12 hours shows that this is the earliest time at which cells that have become heavily labeled during interphase reach mitosis.

At times up to 9 hours, grains over metaphase cells are scattered over the whole cell and are not particularly associated with the chromosomes. At 12 hours and later, the grains are clearly localized in the area occupied by the chromosomes. This suggests that the labeling seen in dividing cells at early times is not due to DNA.

Interpretation of Results

(1) *Rate of incorporation of labeled adenine.* There is no labeling during the irst 30 minutes after adenine injection. This is not due to slow mixing of the njected adenine in the ascites, since Patt, Blackford, and Drallmeier (1953) and Révész and Klein (1954) have shown that when a fluid is injected into the ascites, mixing is complete in two to five minutes.

The average number of grains per labeled interphase cell increases up to six hours (TABLE 1 and FIGURE 6). When this increase is compared with the fall n activity in the cell-free ascitic fluid (FIGURE 2), it appears that the incorporation of adenine into DNA continues at a time when the amount of tracer in the fluid has already fallen to low levels. Taking this observation together with the absence of labeling 30 minutes after injection, we conclude that tracer enters the cells and remains there for an appreciable time before it appears as DNA in the autoradiographs. This time may be occupied by the metabolism of the injected adenine into DNA or a DNA precursor. The metabolic steps through which adenine passes before its incorporation into DNA are unknown. It could be supposed that time is necessary for the polymerization of labeled DNA into a form not removed from the cells during or after fixation.

The failure of the degree of labeling to increase after six hours may be assumed to be due to the exhaustion of labeled precursor taken up by the cells at early times after adenine injection.

(2) *Timing of the mitotic cycle.* If all cells in a population are proceeding at the same rate through a mitotic cycle, and are unsynchronized, the number of cells in any stage of the cycle is proportional to the fraction of the cycle occupied by that stage. If all cells are simultaneously offered a labeled metaboite, and all cells that are in a part of the cycle at which they can incorporate and retain the labeled metabolite do so at the same rate, certain predictions can be made as to the number of cells that will be labeled at later times, and as to the amount of label that they will contain. Let us call the length of the mitotic cycle T, and the length of the period of incorporation of label S. Then the fraction of cells that is labeled in a very short time after the labeled metaboite becomes available is equal to S/T, and 100 per cent of cells will become

labeled at a time equal to T minus S, provided labeled precursor is availabl throughout this period.

If metaphase occupies a very short time compared with T, the maximum amount of label in cells at metaphase will be reached at time S, or at time $S +$ G_2 if an interval occurs between the end of S and metaphase, (this interva has been called G_2 in other materials: Howard and Pelc, 1953; Lajtha *et al* 1954).

We may now examine our experimental observations (FIGURES 5 and 6) making the same assumptions as before, namely, that all cells are proceedin at the same rate, and are unsynchronized, and that the rate of incorporation c label is constant throughout S. The following deductions are made:

(1) Sixty-six per cent of cells are labeled at one hour. If this is considere a short time compared with T, then S/T = 66 per cent of T and T minus S = 34 per cent of T.

(2) All cells (98 per cent) are labeled at six hours. Then T minus S = si hours = 34 per cent of T. Therefore T = 18 hours, and S = 12 hours.

(3) The maximum number of grains per metaphase cell is seen at 12 to 1 hours. The maximum amount of label in any interphase cell is seen at si hours (FIGURE 5). Therefore G_2 = (12 to 15 minus 6) = 6 to 9 hours. I thus appears that S occupies the whole of the first two thirds of interphase and that, during the last one third, no further labeling occurs.

(4) The mitotic index that we measure in these tumors is about 3 per cen If T = 18 hours, mitosis (late prophase to late telophase) then takes about 3 minutes.

(5) The percentage of labeled cells never reaches 100 but comes within a few per cent of it by 4 to 6 hours (FIGURE 6). There is therefore the possibilit that a small proportion of cells remain unlabeled. This proportion, howeve cannot be more than about 10 per cent if the criterion of labeling is correct.

The estimate of 18 hours for T is supported by other features of the data If we make the further assumption that the amount of labeled material presen in a cell is reduced only by its partition between two daughter cells at mitosi we should expect the average amount of label to fall by one half at each mitoti cycle, provided no further labeling occurred. The average number of grain per interphase cell is at a maximum at four to six hours and reaches half th value at about 20 hours, giving an estimate of about 15 hours for T. Similarly the number of grains per metaphase cell is halved about 15 hours after its pea value is observed (FIGURE 6).

To assess the reliability of the estimate of the length of T, we may conside what are the shortest and longest times that are compatible with the data

(1) *Shortest estimate of* T. It may be argued that the criterion of labelin that has been used is incorrect and that less than 66 per cent of cells becom labeled by one hour. If so, S is a smaller proportion of T and, if T minus is not more than six hours, T would be correspondingly decreased. Sinc a progressive increase in the number of grains counted over the most heavil labeled cells is observed up to four hours, we conclude that S cannot be shorte than four hours. This argument yields a minimum estimate for T of 10 hour

(2) *Longest estimate of* T. If more than 66 per cent of cells become labeled t 1 hour, the estimate for T would be longer than 18 hours. Considering the our points in TABLE 1 and in FIGURE 6 for percentage of labeled cells at one, ne and one-half, and two hours, and allowing 10 per cent for sampling error, t appears that the one-hour value can be supposed to be as high as 75 per cent, ut this seems to be the maximum likely figure. This value yields an estimate f 24 hours for T.

It can be argued that if our criterion of labeling is in error, the percentage of abeled cells may be lower in reality than that shown in FIGURE 6. Whatever riterion of labeling is taken, the percentage of labeled cells does not increase fter six hours. This could be explained, however, by exhaustion of the supply f labeled DNA precursor. These considerations might provide grounds for upposing that T minus S could be longer than six hours and, therefore, that a ime gap of unknown length might occur between division and synthesis. This argument, however, faces the difficulty that, whatever criterion of labeling s used, the rate of increase in percentage of labeled cells is high between one nd six hours, of the order of 10 per cent per hour. Such a rate of entry of cells nto synthesis could be explained only by a short time for T, a value below 5 hours agreeing best with the observations.

From these considerations, we conclude that the length of the mitotic cycle, stimated on the assumption that all cells are proceeding through a mitotic ycle at the same rate and are unsynchronized, is 15 to 18 hours. Reasons ave been given for believing that the radioactive label observed in the cells s DNA. We therefore conclude that S is a period during which adenine is ncorporated into DNA, and that this incorporation occurs during a 12-hour eriod ending about six hours before metaphase.

Discussion

The mean generation time in the Ehrlich ascites tumor has been estimated y Klein and Révész (1953) from measurements of the rate of increase in cell umber. It was found to depend on the number of cells inoculated and on he age of the tumor. From Klein and Révész' text FIGURE 3, the mean generation time in tumors such as we have studied (*i.e.*, at five days' growth after n inoculum of approximately 12 × 10^6 cells) is about 35 hours. If all cells re viable, this represents the mean duration of the mitotic cycle. Our data ead to a most likely estimate of 15 to 18 hours and a maximum estimate of 24 ours for the mitotic cycle. There are obvious reasons why we cannot assume hat Klein and Révész' estimate should apply narrowly to the tumors that we ave studied (different genetic constitution of host mice; possibility of a change n the growth characteristics of the tumor subline; the large differences between individual mice observed by Klein and Révész). It is nevertheless of nterest to assume that, if all cells are viable, a closer agreement between the stimates is to be expected, and to consider the implication that some cells die.

If the Schrek test is taken as the criterion of cell viability, very few dead ells are seen in the Ehrlich ascites tumor until the terminal growth stages Klein and Révész, 1953). Since, however, we do not know the rate at which dead cells disappear from the population, the number seen gives no measu of the rate of cell death. On the other hand, there are several indications that considerable number of cells are nonviable or at least unable to complete d vision. Levan and Hauschka (1952) and Makino and Kano (1953) have con cluded from chromosome counts at metaphase that the growth of certain ascite tumors, including the Ehrlich, is maintained by the division of only a part the cell population, the stem cells, the remainder of the population bein unable to divide successfully. This stemcell theory is supported by Richard (1955) observation that the amount of DNA measured by Feulgen photometr shows, at metaphase, a wide spread of values comparable to the spread chromosome numbers, but that, at telophase, the values fall within a muc narrower range. This indicates that many metaphase cells do not procee to telophase. Laznitski (1953) had already concluded, in the S 37 sarcoma an T 2146 tumor growing in the ascites form, that many cells that reach metapha fail to cleave.

The stemcell theory proposes that a fraction of the cell population is effe tively outside the mitotic cycle. We observe that a large proportion of th cell population becomes labeled in a short time, and that the number of cel that do not become labeled must be small. Our results suggest, therefore, tha cells that leave the dividing population do so shortly after having becom labeled.

The Ehrlich tumor is tetraploid, two thirds of metaphase cells having b tween 75 and 84 chromosomes (Hauschka and Levan, 1951), The amoun of DNA per cell is twice that found in nontumourous cells (Goldberg, Klei and Klein, 1950) and the DNA per nucleus is twice that found in the DB mouse ascites lymphoma (Leutchenberger, Klein, and Klein, 1952), which ha a diploid chromosome number. Values for the average DNA content per ce of appreciably more than the tetraploid have not been observed biochemicall This suggests that few cells having the octoploid amount can be present, ar it follows that an increase in the amount of DNA takes place shortly befo division. Richards, Walker, and Deeley, elsewhere in this monograph, in terpret their photometric results to mean that increment in Feulgen-stainin material also occurs towards the end of interphase.

We observe that labeling of DNA proceeds throughout the greater part interphase, and that a period of nonlabeling occurs at the end of interphas In view of the biochemical and photometric findings, we may suppose that th labeling that we observe is not associated with an increase in amount of DN and, further, that at the time this increase is occurring, there is a much r duced rate of incorporation of new adenine.

Summary

The autoradiograph method has been used to study the uptake of [8-^{14}C adenine into DNA of Ehrlich ascites tumor cells. Ribonucleic acid was r moved from smears by ribonuclease digestion. An estimate of the amount labeled DNA in individual cells was made by counting the number of phot graphic grains developed in the area of film covering the cells, and subtractin background.

From the proportion of interphase cells that become labeled at different mes after injection of adenine, and from the amount of labeled DNA that terphase and dividing cells contain, it is concluded that: (1) the great marity of cells incorporate adenine into DNA; (2) the mitotic cycle in those cells at divide is 15 to 18 hours, the maximum estimate consistent with the data ing 24 hours; and (3) incorporation of adenine into DNA takes place in terphase, and an interval of about six hours occurs between synthesis and vision.

The implications of these conclusions are discussed with regard to the growth the tumor and the synthesis of DNA.

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Discussion of the Paper

Doctor David Pressman (*Roswell Park Memorial Institute, Buffalo, N. Y.*): t is quite clear from the paper by Doctors Hornsey and Howard and from ther papers preceding it in this monograph that the incorporation into nuclear NA of desoxyribose as determined with the C^{14} labeled sugar, of phosphate s determined by incorporation of P^{32} phosphorus or adenine as given by the corporation of C^{14} adenine takes place at different times for these three differnt components. The actual formation of DNA is thus more complex than ould appear from the incorporation of any one of these components. I ink that further discussion would be much more valuable if it were to come om workers who are actively engaged in such investigations.

DOCTOR G. B. BROWN (*Sloan-Kettering Institute, New York, N. Y.*): Som recent developments in our laboratory may be pertinent to the interpretation of the incorporations of adenine in experiments such as those described b Doctors Hornsey and Howard. Studies by Doctors Roll and Weinfeld wit multiple-labeled nucleotides have confirmed that the pyrimidine-ribose moieti (pyrimidine nucleosides) are incoporated as single units. With similar purin derivatives, however, there is a considerable transfer of purines to new ribose or a "transpurination" type of reaction. The available evidence demonstrates only that such a reaction occurs at some stage between the injection a nucleotide and the appearance of its components in a polynucleotide. However, the postulation of a "transpurination" between some "active" adenin derivative and the "back-bone" of a polynucleotide offers certain advantag for reinterpretation of some of the existing data that show differences betwee the incorporations observed *in vivo* for exogenous purine and small purine precursors, such as glycine and formate.

Any such "transpurination" of purines into DNA when that nucleic acid in an active metabolic stage might represent an incorporation that was ind pendent of total synthesis, and would complicate interpretations of incorporation data.

Doctors Pelc's first experiments with adenine-8-C^{14} were made during a vis to the Sloan-Kettering Laboratories, and I am sorry to have to add now th word of caution. It do feel that our recent data necessitate the consideratio of the possibility that the incorporation of adenine does not necessarily repr sent total synthesis, and that this may influence the interpretations of resul such as those reported by Doctors Hornsey and Howard.

RADIATION EFFECTS ON ASCITES TUMOR CHROMOSOMES*

By Alan D. Conger
Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.

We have been interested in the ascites tumor as a material for radiation tudies, and have sought for methods by which quantitative radiation experiments could be done with it. Our primary object has been to analyze effects n the treated cells themselves, and so the experiments and methods were esigned, in so far as possible, to minimize complications that might arise rom secondary reactions, such as host immunity response, and the like. Three cellular effects of radiation have been studied quantitatively with the Ehrlich ascites tumor: (1) chromosomal damage, (2) mitotic inhibition, and 3) cell death as measured by suppression in cell population increase. Corelations among the three effects can be demonstrated. In a general way, hese three radiation effects showed the same response in ascites tumors as ad been found with the classical materials used for such analyses, as *Vicia* nd *Tradescantia*[1, 2] for chromosomal damage, grasshopper neuroblasts for mitotic inhibition,[3] and microorganisms for cell death.[4]

Chromosomal damage is readily determined by observation of the fraction of naphases of division that are abnormal, that is, anaphases that display chromoomal bridges, chromosomal fragments, or both. The observation is simple nd unequivocal though, for radiation experiments, some disturbance may be aused by variability in the spontaneous abnormality rate. It is, in fact, bundantly clear that many tumor strains are so prolifically and variably aberant that they are unsuited to experimentation of this sort. Our experience is imited to the Ehrlich ascites tumor, but in this we have found considerable differences in the frequency of spontaneously abnormal anaphases in four different substrains, all originally derived from a single source (Klein). The sublines were different in growth rate and other factors as well. The subline Ehrlich umor we use, kindly supplied by Doctor Theodore S. Hauschka, is generally bout 85 to 90 per cent normal.

Most of the experiments have been with *in vitro* irradiation because it alowed experimentation with a diversity of radiations as well as with modifying gents. In a typical experiment, a pooled sample of ascitic fluid diluted with bout one fourth its volume of heparinized Tyrode's solution is subdivided nto lots and given various doses of radiation, often with adjunctive treatments.

Shortly after irradiation, each lot is inoculated into animals for subsequent growth. Sampling at successive intervals after irradiation shows (FIGURE 1) n initially rapid decline in the fraction of normal (aberration-free) anaphases o a minimum, then a slow recovery until the cell population eventually returns o the unirradiated value. In later experiments, the period from 0 to 48 hours was examined in greater detail—every four hours—to see if anaphase normalcy declined in the smooth fashion indicated by the extrapolated lines from 0 to 24 hours in FIGURE 1. It was found that there was a very rapid initial fall normalcy to a minimum at about six hours, then a leveling off or even sligh rise, followed by a second smooth decline in normalcy to the minimum at abou 24 to 48 hours shown in FIGURE 1. We are inclined to think that the fir rapid minimum represents that small proportion of cells that were in propha at the time of irradiation and continued on to enter anaphase without dela The second minimum, at 24 hours in the example shown, represents the bulk the cell population that was in interphase when irradiated and is dividing f the first time. The gradual return to normalcy shows that the cytological

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* Work performed under contract W-7405-eng-26 for the Atomic Energy Commission.

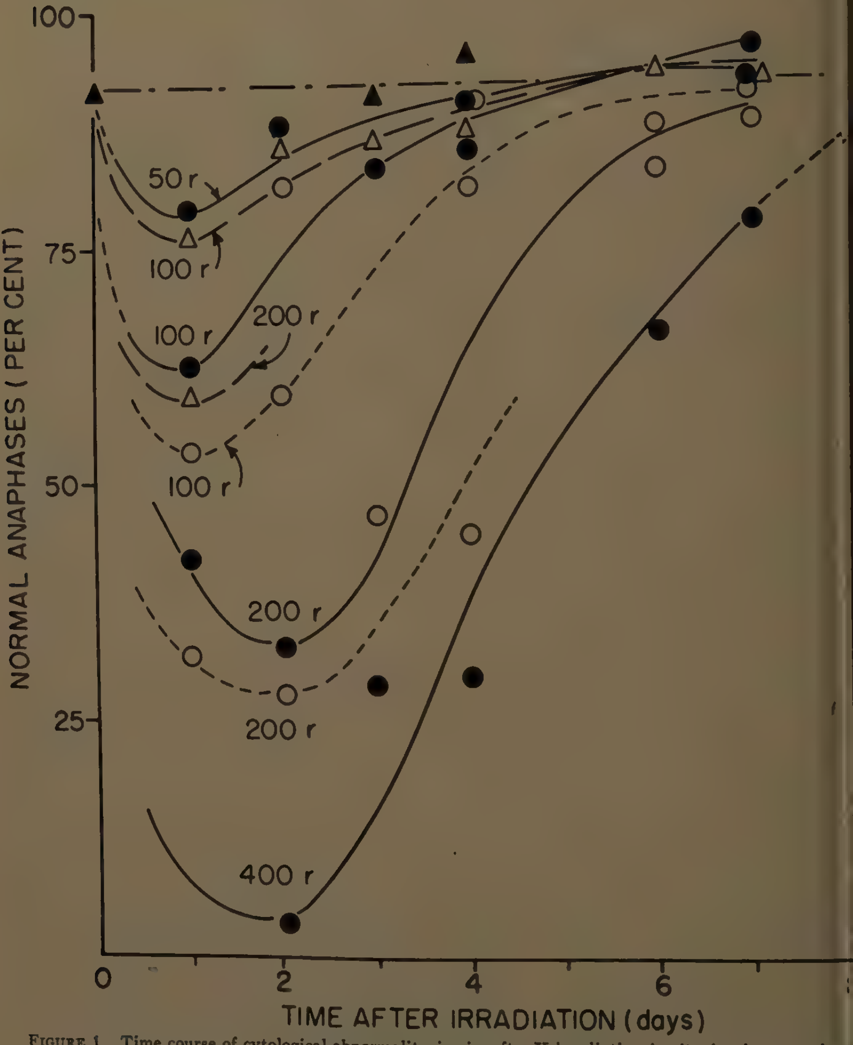

FIGURE 1. Time course of cytological abnormality *in vivo* after X irradiation *in vitro* in nitrogen, air, a oxygen. ▲ = control, △ — — = nitrogen, ● —— = air, ○ - - - = oxygen.

normal cells are not contributing to the continuing population, but are elimi- ted as they divide, and so die off.

Apparently, some cells may delay entry into their first division for as long as e or six days, which accounts for the abnormal divisions, in excess of control, en at the long times after irradiation. Most abnormal cells seem to divide ly once, as judged by the rarity of divisions displaying old micronuclei or agments generated by a previous division. Increasing the dose causes a eater decline and a subsequently longer period for recovery. This general sponse holds for radiation experiments done under the most varied conditions, e difference in conditions being reflected by an alteration in the time scale, it not the shape, of the depression and recovery curve. For example, the inimum of anaphase normalcy from *in vitro* irradiations is reached at about to 48 hours after irradiation, depending on the *in vitro* conditions but, with *vivo* irradiation, the minimum may occur as early as six hours. The delay om *in vitro* irradiations is greater than that from *in vivo*, of course, because the *vitro* handling itself causes initially a division delay in addition to that caused the radiation.

Although the time-response curve for cytological abnormality reveals a imber of interesting and important features of cytological response to radia- on, it is not these aspects we are primarily interested in, but rather in how fect is related to dose of radiation and how modifying agents can alter the se response. It has been pointed out already that the major minimum of rmalcy, occurring at about 24 hours in the example of FIGURE 1, represents e large proportion of tumor cells that was in interphase at the time of radia- on and is now dividing for the first time. Over a considerable dose range, so ng as dose is not too great, the time at which these minima occur is approxi- ately independent of dose, and the minima are broad enough to overlap. naphase abnormalcy is, therefore, a reliable criterion of effect. The frac- on of normal anaphases taken from FIGURE 1 at the time of minimum normalcy shown in FIGURE 2. At each point, not less than 200 anaphases were ob- rved. The fraction of normal anaphases shows an exponential decrease with se, up to such dose that only about 10 to 15 per cent of the cells are still rmal. With greater effect, normalcy begins to decline more rapidly. The iginal intent of the experiment was to measure the effect of anoxia on the diosensitivity of the tumor cells,[5, 6] and the effect is easily measurable from e dose curves. Not always is the fit of the points to the line as good as is und here. In other experiments, there was considerably more scatter.

Perhaps most surprising is the remarkable radiosensitivity of the cells. A se of only 170 r *in vitro* in air is sufficient to depress normalcy 50 per cent. another experiment, about 100 r of X rays or 45 rep of fast neutrons *in vitro* oxygen caused 50 per cent abnormal cells. When six-day-old fluid was -irradiated *in vivo*, approximately 200 r sufficed. In terms of more familiar perience with the radiosensitivity of tumors, based mostly on solid tumors radiated *in vivo*, the sensitivity shown here may be so surprising as to be un- elievable. It should be emphasized again that what is measured by these periments is the response of the tumor cells themselves, independent, in the ain, of any secondary reaction of the host Furthermore, and equally important, what is observed is the response of the original population of irradiate tumor cells dividing for the first time. If, instead of examining the damag to the original population on the first day after treatment—the curve for 100 in air of FIGURE 1 will serve as an example for this—we had waited until th sixth day, then the amount of damage, *i.e.*, apparent radiosensitivity, woul

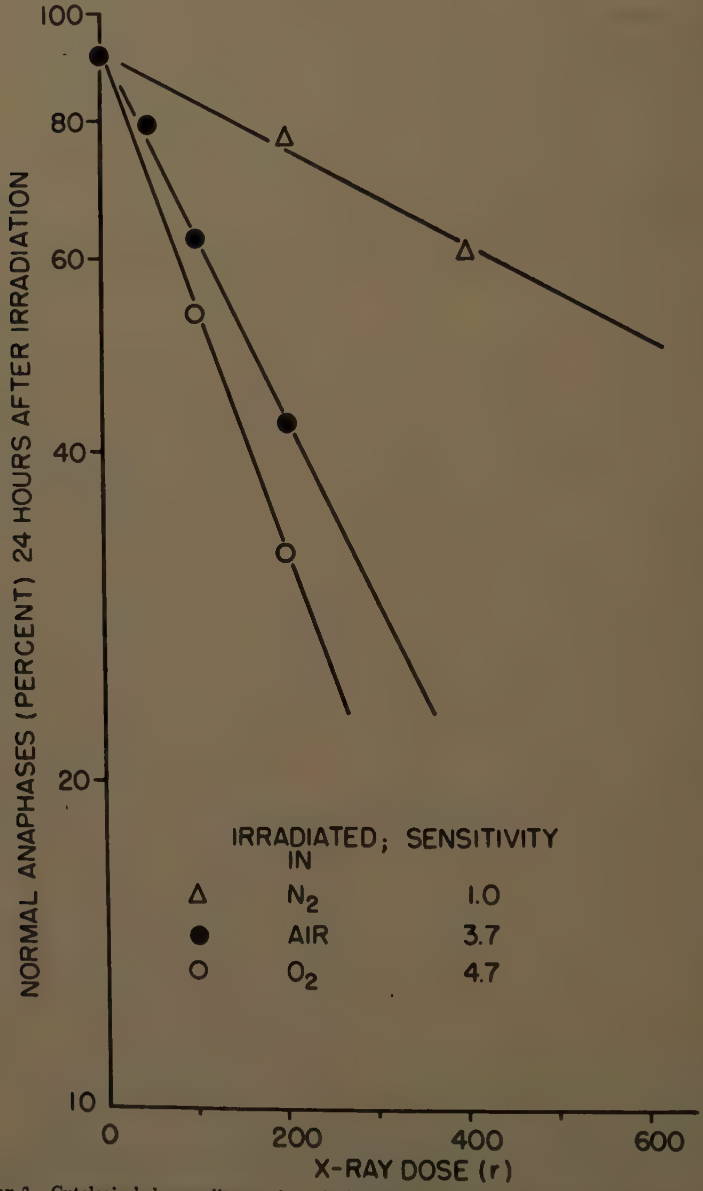

FIGURE 2. Cytological abnormality, at time of minimum normalcy, as a function of X-ray dose *in vitro*.

ave been considerably less. On the sixth day, however, we should have been bserving the effect, not on the originally irradiated population, but on that action of damage that still persisted in a replacement population after most f the affected cells had been eliminated, and the surviving undamaged cells ad undergone several duplications. Such a late observation would be doubly eceiving. By elimination, it would reduce the total number of damaged cells een and, by allowing duplication of unaffected cells, it would reduce the proortion of damaged cells in the total population examined.

The second assay method for radiation damage has been the familiar one of ose *versus* cell survival, as measured by subsequent increase in cell population ze. Diluted ascitic fluid was irradiated *in vitro* with increasing doses of radiaon, as was done in the cytological experiments, except that, for each dose, a easured number of cells was inoculated into a mouse. Five days later— hich is at about the midpoint of the period of exponential growth of the poputiontion —the total number of cells per animal was counted.[7] FIGURE 3 illustrates he effect of radiation on cell population increase in five days of growth after radiation and inoculation. The figures are averages from the four to six nimals used at each dose. During this time, the unirradiated sample has inreased 22 times, equivalent to four and one-half doublings. Population size on he fifth day decreases exponentially with dose over the dose range tested. Iere again, as with the cytological experiments, this relation was valid down nly to about 15 per cent survival. With greater effect, cell number fell off ore rapidly, probably owing to secondary reactions unrelated to the primary ffect of the radiation on the tumor cells. The decreased growth in the irraditd samples cannot be accounted for by suppression of division caused by the radiation, but must be owing mostly to killing of the cells. At even the rgest dose, division is inhibited only 12 hours more than the control; such a ivision delay, even with generous allowance, could account for not more than ne third of the decrease actually observed. The experiment is dependent on here being a similarity in the five-day-growth curves for all the doses analyzed. n order to achieve this in practice, it is necessary that the inoculum be not so rge that growth declines appreciably because of early saturation of the host, or so small that it suffers an extensive lag or even regression because of host esponses, within the five days of growth. For these experiments, this meant hat the inoculum size was held between approximately 2 to 20 × 10^6 viable ells. Such restriction minimizes the confusion that may arise from reactions nrelated to the primary one of radiation on the original cells.

It is worth while to compare the radiosensitivity in terms of survival from his experiment on cell growth with the former one on cytological effect. A ose of about 320 r *in vitro* in air depresses survival 50 per cent, as measured y five-day growth. A dose of 170 r sufficed to render 50 per cent of the cells ytologically abnormal, *i.e.*, genetically inviable (FIGURE 2). The complicaions in making such a cytological-survival intercomparison are considerable. actors such as estimation of total cytological abnormality by integration over he total period of its expression, allowance for survival of some abnormal cells ecause the tumor strain is a subtetraploid, and the fact that the two experients compared were not done at the same time, must all be considered. If,

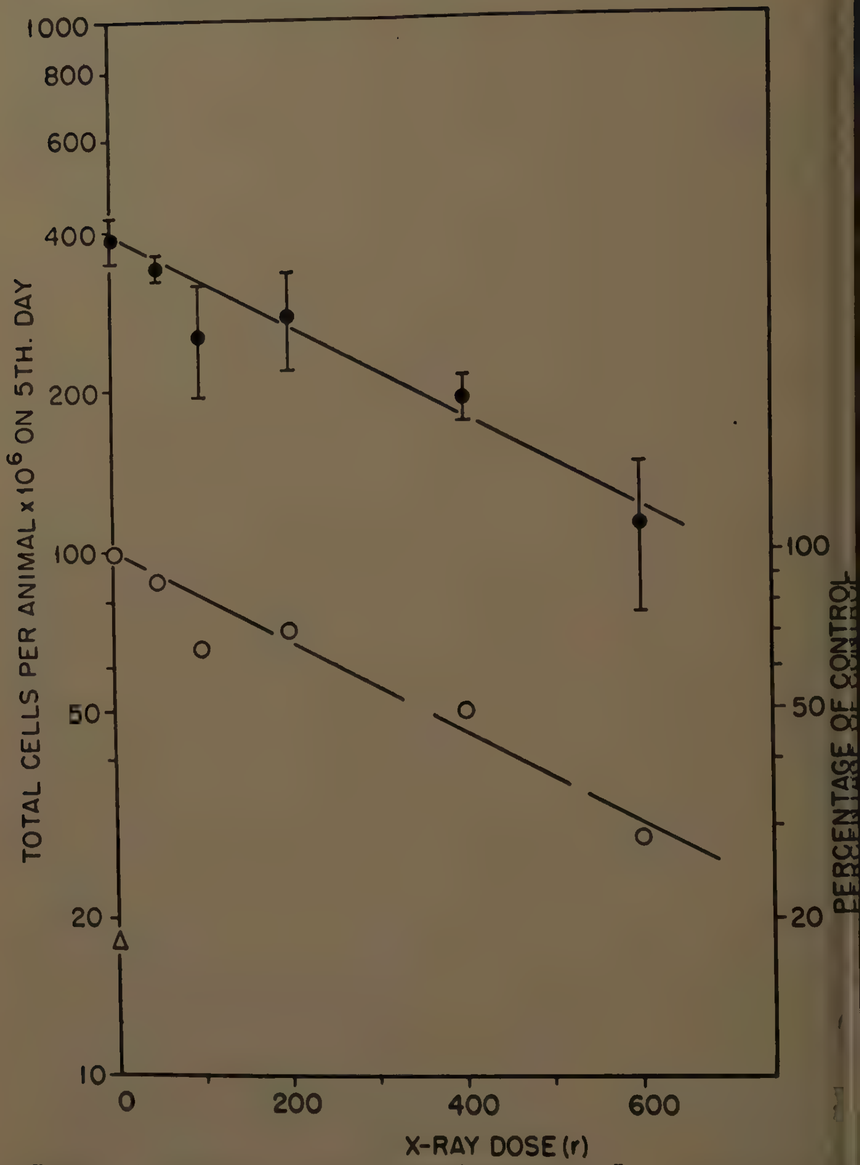

FIGURE 3. Five-day growth of tumor after X irradiation *in vitro* in air. ● = Total cells per animal, fi
day ± S.D.; △ = inoculated on day zero; ○ = percentage of control, fifth day.

in spite of these objections, the sensitivity as measured by both criteria is hig
then it does seem justified to say the cells are quite radiosensitive.

Mitotic inhibition, measured by the percentage of cells in division, was o
served in the same samples studied for anaphase abnormalities, and a clo
temporal correlation was found for the period of initial decline and maximu

ect. The minima of anaphase normality and of division occurred at about e same time. This correlation seemed to hold whether the response were pid, as with the *in vivo* treatments, or up to six times as slow in the *in vitro* periments. Mitotic inhibition in ascites was quite similar to that found in e well-known grasshopper material, but could be followed for a longer time, ., more divisions. Doctor W. St. Amand, of this laboratory, has very kindly lowed me to use as an illustration some of his results on mitotic inhibition om irradiation *in vivo*, shown in FIGURE 4. Each curve is an average from o animals irradiated six days after inoculation. In these results, the per- ntage of cells in division (mitotic index) was initially depressed by an amount d for a time dependent on radiation dose. The amount and duration of the pression are related directly to the dose, but the rapidity of their appearance inversely related to it. This agrees quite well with the grasshopper results om the same kind of experiment. Following the initial inhibition, division covers the normal rate only to overshoot it and then, although the figure es not extend far enough to show it, continues on as a damped oscillation out the unirradiated value. Correlation with anaphase abnormality could t be shown for the later period of continued oscillation. Mitotic inhibition, wever, unlike anaphase abnormalities resulting from chromosome breakage, ry clearly can be caused by a large number of factors other than radiation. me of these factors are age of inoculum, time of day, difference between ani- als, shock of handling, and fluid withdrawal; and certainly some of them will encountered as uncontrolled variables in the course of a radiation experi- ent. The difficulty of eliminating such variables is considerable. In general, though mitotic inhibition displays many features of intrinsic interest, in

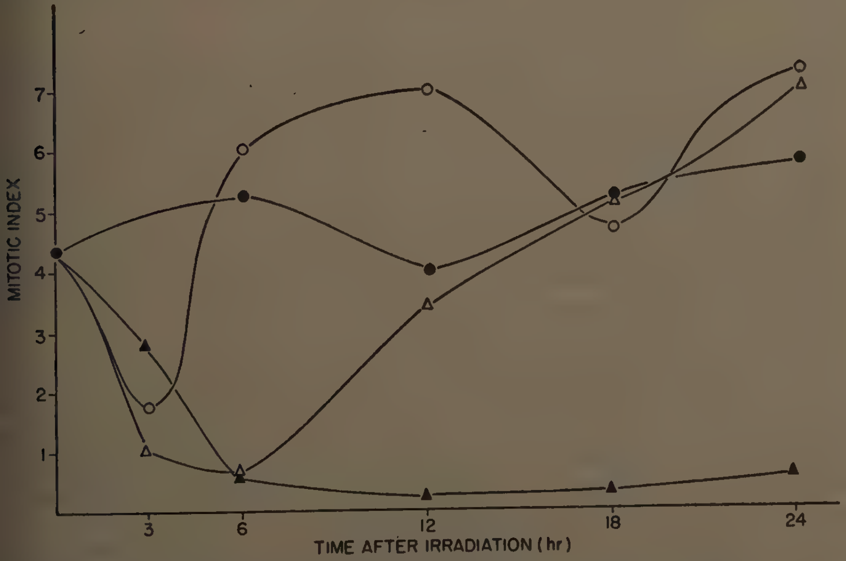

FIGURE 4. Time course of mitotic inhibition after *in vivo* irradiation of tumor inoculated 6 days previously. itotic index = percentage of cells in division. ● = control, ○ = 100 r, △ = 200 r, ▲ = 400 r.

ascites tumors it has been a less reliable criterion of radiation damage than anaphase abnormality.

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Discussion of the Paper

Doctor Arnold H. Sparrow (*Biology Department, Brookhaven Nation Laboratory, Upton, N. Y.*): It is apparent from the work presented by Doct Conger and several of the previous speakers that the ascites tumor cell len itself well to the study of changes in chromosome number, chromosome mo phology, and mitotic rate, as well as to quantitative studies of chromosom aberration frequency. Since these are all aspects of chromosome behavi that are influenced by ionizing radiation, it is only natural that the cytologi should take advantage of this material for radiation studies.

The excellent data presented by Doctor Conger illustrate very well that r liable quantitative methods have been applied to the analysis of radiati effects on ascites tumor cells. His dose curves are very impressive and ce tainly suggest that further valuable contributions can be made along the lines.

The mitotic inhibition data are of special interest to me, as is the recove of the mitotic rate and the manner in which it overshoots the normal number divisions after certain dosages. It is questionable, however, whether the tot number of divisions occurring in a given interval following low doses of radi tion would exceed the number found in controls if all divisions occurring we integrated for the whole period.

The phenomenon of mitotic inhibition is a familiar one to many cytologis but its cause is not well understood. It may be due to one or more of the f lowing possibilities: (1) inhibition of synthesis of DNA or some other essenti component of the nucleus; (2) production of a cell poison or antimitotic su stance; (3) chromosome breakage and aberration.

The choice between these possibilities is difficult to make, but I should li to comment on the possible relationship between chromosome breakage a mitotic inhibition.

We have shown in *Trillium* that the duration of the inhibition is related the amount of chromosome breakage induced.[1] This is true even when no lo of chromatin due to lagging of acentric fragments has occurred. This wou indicate that mere breakage itself might be a factor in mitotic inhibition ev for nuclei that have not yet passed through an anaphase stage after exposu to radiation.

This could mean either that breaks *per se* cause an interference with the normal physiology of the nucleus (the effect might be compared to the well-known henomenon of position effect) or it could mean that breakage and inhibition re both due to radiation damage at the molecular level; *e.g.*, in the nucleo-rotein molecule, and are not otherwise causally related. Mitotic inhibition ccurring after the first cell division has been completed is much more likely to e due to loss of genetic material. Other factors, however, such as physiolog-cal effects could also be involved.

Although this section, PART II, is entitled "Ascites Tumors as Assay Tools," erhaps studies of this nature can also contribute information concerning the undamental cell biology of ascites tumors. I should like to comment on some f these possibilities.

It has been shown in my laboratory and elsewhere that, in general, poly-loid plants have a higher tolerance to radiation than diploids. As far as I now, this has not been tested in ascites tumors, but I am sure suitable diploid nd tetraploid strains exist. In this connection, it would be interesting to see whether radiation-resistant strains (see the paper by Lettré, in this monograph) ave more chromosomes than less resistant strains. It would also be worth while to find out whether repeated exposures to ionizing radiation might not end to hasten the change from diploid to tetraploidy since radiation is known o produce polyploid cells[2] and, once produced, they could be expected to be more tolerant to radiation and hence should have a selective advantage over iploids.

The use of radiation in genetic studies has led to an area of investigation often eferred to as radiation genetics. The application of radiation in classical enetics has been a very fruitful field of research. Perhaps radiations can also e successfully used in the studies of the genetics of somatic clonal lines. The ollowing questions could be asked: (1) Can genetic changes, *e.g.*, immunity eactions, be induced without a change in chromosome number, *i.e.*, by point mutation or loss of genetic material? (2) Would clones established after ir-adiation from one or a few cells show a greater number of variants then is normally found in control lines? (3) If so, then would radiation accelerate he transformation of solid tumors to the free cell ascites type?

In conclusion, I should like to say that there is every reason to expect and ope that radiobiology and the biology of ascites tumors will each lead to mu-ually worthwhile advances in our knowledge of fundamental problems of both normal and abnormal growth.

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STUDY OF METASTASIS WITH THE AID OF LABELED ASCITE TUMOR CELLS

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Introduction

The study of metastasis is of obvious clinical significance. The majority deaths from neoplastic disorders are due to metastatic infiltration of vital o gans. Metastatic capacity is one of the easily recognizable characteristics neoplastic cells, and thus it may become an important tool in the hands of th biologist and population geneticist. Koch (1939) developed highly metastat sublines by transplanting metastatic cells serially. Klein (1955) showed tha in at least one tumor, conversion from the solid to the ascitic form is correlate with increased ability to form metastasis. Kaziwara and Hauschka (1955) in jected a mixed population of Ehrlich carcinoma cells subcutaneously into th tails of mice. After a period of growth, the tails were amputated together wit the local tumor. In the subsequently developing metastasis, the polyploi elements appeared to be selected out. Watanabe (1954) found correlation be tween capacity to form metastasis and heterologous transplantability. It a pears that for the cell population geneticist, metastasis, as an assay tool, offer advantages very similar to those of the ascites transformation. Nevertheless relatively little work has been done on the phenomenon of metastasis. Th may be due to the lack of reliable quantitative methods.

The aim of this study was to develop quantitative methods for the invest gation of the distribution and fate of embolic tumor cells, particularly in th early stages, before routine pathologic methods become applicable.

Factors involved in spontaneous blood-borne metastasis formation can b divided into the following phases:

Release of metastatic cells from the primary tumor. Coman and his associate (review, 1951) concluded that local invasiveness depends on loss of cellula adhesiveness, which is related to local calcium deficiency. Tyler (1947) an Weiss (1947, 1950) suggested that cell surface antigens and autoantibodies ma be involved in the cohesion of cells. Spiegel (1955) raised the question wheth loss of such autoantibodies or antigens could be responsible for release of meta static cells. Baserga and Baum (1955) called attention to the significance the location of the primary tumor in showing that a transplantable sarcom that does not metastasize when implanted into the subcutaneous tissue of th trunk produces large numbers of metastases after subcutaneous inoculation int the tail.

Transport of embolic cells and lodging of same in organs. Coman (1953) be lieves that anatomical and hemodynamic factors are mainly responsible for th distribution of metastases. Coman, Eisenberg, and McCutcheon (1949) foun at V_2 carcinoma which spontaneously metastasizes only to the lungs, and rms metastasis in most organs after injection of tumor-cell suspensions into e left heart. DeLong and Coman (1950) implanted various tumors directly to abdominal organs and muscle in several species. No important differences ere observed in growth potentiality in these organs. On the other hand, Lucké, reedis, Woo, Brewick, and Nowell (1952) found significant differences in the bility of the lungs and the liver to support growth of V_2 carcinoma. Similar onclusions were reached on the basis of 154 human autopsy cases with liver nd lung metastases.

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Local growth of metastatic cells and invasion of surrounding tissues. Iwasaki 1915–16), Takahashi (1915), Pearce and Brown (1923), Warren and Gates 1936), Willis (1952), and many other investigators emphasized that the marity of embolic tumor cells fail to establish themselves as metastatic tumors. ugarbaker (1952) compared metastatic distribution in rats following injection f suspensions from various tumors into the left ventricle. It appeared that ach tumor establishes a characteristic metastatic pattern. Willis (1952) summarized the evidence (mainly on the basis of clinical findings) in favor of the nportance of local tumor-organ relationship factors in the establishment of haracteristic metastatic patterns. The latter views, most often referred to s the "seed-soil" hypothesis, are in obvious contrast with the "mechanical" heory of Coman (1953) and others.

In research on experimental metastasis it is important to be able to study he above phases independently from each other. Elimination and standardiation of the first phase (release) can be accomplished easily by injecting tumorell suspensions into the vascular system. For this purpose, ascites tumors ffer obvious advantages: (1) known numbers of tumor cells can be inoculated; 2) little interstitial tissue is inoculated with the tumor cells; (3) no mechanical isintegration procedure is necessary to produce tumor cell suspensions, and hus no injured cells are injected. Révész (1955) demonstrated that inoculaion of injured cells may alter growth patterns of neoplastic cells. Zeidman, cCutcheon, and Coman (1950) found that in a suspension of tumor cells sed by them, only 6 per cent of the cells were viable according to the trypan lue uptake test; finally (4) no large aggregates of cells are injected. Baserga nd Saffiotti (1955) reviewed the controversy in the literature as to whether ingle tumor cells or large aggregates of cells are more successful in establishing etastatic tumors. Certainly ascites tumors offer more uniform suspensions han do disintegrated solid tumors. Warren and Gates (1936) and Pomeroy 1954) were the first, to our knowledge, to use ascites tumors in the study of etastasis.

Most students of the problems of metastasis relied simply on macroscopic bservation of metastatic tumors. Thus, no distinction could be made between he phase of transport and distribution and the phase of local growth and inasion. Little knowledge is available on the early distribution of embolic umor cells. Coman, DeLong, and McCutcheon (1951) injected into the left eart of rabbits suspensions of fixed, stained tumor cells and studied their disribution in histologic sections. Goldie, Jeffris, Jones, and Walker (1953) injected mashed organs into recipient mice and used the developing ascites tumo as an indication of the presence of metastatic tumor cells in these organs. Th growth rate of the ascites tumor may be at least a semiquantitative measure o the number of tumor cells in these organs. Because of the findings of Révés (1955) on the effect of injured cells on the growth rate of ascites tumors, thi method can probably not be regarded as strictly quantitative. We have attempted to label ascites tumor cells with P^{32} and quinacrine and follow the fate by measuring radioactivity of the various biochemical fractions and fluorescence under the ultraviolet microscope.

Materials and Methods

Webster Swiss, DBA/2, Strong A, AK, C_{57}Bl/6, and C3H male mice of ou own colony were used in these experiments. All animals weighed 20 to 24 gm at the beginning of the experiment. The Ehrlich ascites carcinoma was obtained originally from Doctors G. and E. Klein, Karolinska Institute, Stockholm, Sweden. Sa 37 and Sa 180 were obtained in ascites form from Doctor H Goldie, Meharry Medical College, Nashville, Tenn. A total of 2.5 × 1 tumor cells were inoculated intraperitoneally into Webster Swiss mice and, 7 hours later, P^{32} was injected as sodium phosphate intraperitoneally. Th treatment was repeated for a total of five days. Forty-eight hours after th last injection, the animals were sacrificed and the ascitic fluid harvested. T avoid clotting, 1 ml. of 0.1 M sodium oxalate solution was added to each 10 m

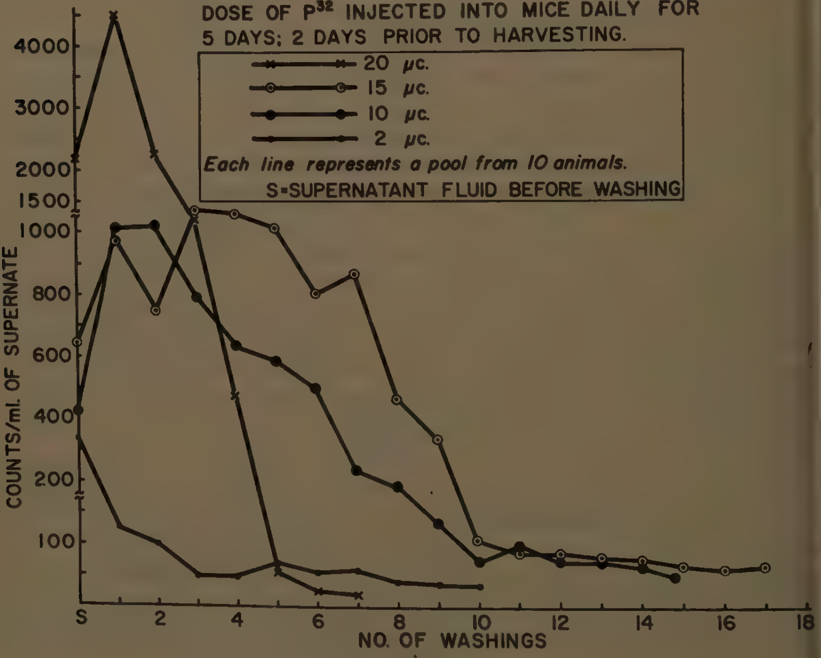


FIGURE 1

TABLE 1

EFFECT OF WASHING ON RADIOACTIVITY OF P^{32}-LABELED EHRLICH ASCITES CELLS

Treatment*	Counts per minute per ml. of cells			
	First generation		Second generation	
	Before washing	After 10 washings	Before washing	After 5 washings
5 μc/kg. i.p. daily (10 animals)	893	325	36	—
.0 μc/animal i.p. daily (10 animals)	11,856	11,052	70	55
0.0 μc/animal i.p. daily (20 animals)	89,665	27,700	85	60
5.0 μc/animal i.p. daily (20 animals)	134,896	79,894	96	73
0.0 μc/animal i.p. daily (20 animals)	112,067	83,665	85	90

* P^{32} injections were given for five days, beginning 72 hrs. after the i.p. inoculation of 2.5 × 10^6 Ehrlich scites tumor cells. Tumor cells were harvested 10 days after inoculation.

f ascitic fluid. This procedure is essentially a modification of that used by ettré, Rossak, and Wrba (1953). The ascites was successively washed with ocke's solution. FIGURE 1 shows the radioactivity of the supernate after nitial centrifugation and after each washing. The groups shown received laily doses of P^{32} ranging from 2 to 20 microcuries. The fact that the activity f the first washing fluids is higher than that of the original ascitic supernate ndicates that some labeled compounds are actually removed from the cells. Vith 10 washings, at the most, a plateau of supernate activity was reached. ne ml. sample of packed cells was obtained before and after the 10th washing, nd its radioactivity determined. Similarly, labeled cells were inoculated intraperitoneally into recipient mice and, after 10 days, a "second transplant eneration" of cells was harvested, washed, and studied as described above. n the "second transplant generation" cells, a plateau was reached after, at nost, five washings. TABLE 1 summarizes the results. It appears that, in the 'first transplant generation," washing substantially reduces the radioactivity f the cells. In the "second transplant generation," less reduction is present. t was found that, for the continuous recording of the presence of labeled tumor ells in the blood stream (as described below), 15 microcuries per day is the opimal dose. This was used in all experiments to be reported. Two and onealf million tumor cells, labeled with doses of 2 to 20 microcuries per day, were njected into recipient mice. Increase in weight and ascitic tumor cells was bserved and compared with that of control animals receiving nonlabeled cells. en days later, the animals were sacrificed, the ascites harvested, and the same umber of cells injected into a second series of mice. It appears from TABLE 2 hat growth rate of P^{32}-labeled cells does not differ significantly from that of ontrol cells in either the first or the second "transplant generation."

The level of circulating, labeled, ascites tumor cells was continuously recorded y the following procedure. Mice were anesthetized with 150 mg./kg. hexoarbital sodium (Evipal Sodium) administered intraperitoneally. They were astened to a mouse board, and the tails were placed into the groove of a speifically constructed lead shield (FIGURE 2), into which a shielded Gieger counter vas fitted above the tail. The latter was connected to a radiation rate meter

TABLE 2

EFFECT OF LABELING WITH P^{32} AND QUINACRINE ON GROWTH OF THE EHRLICH ASCITES TUMOR. MEASUREMENTS TAKEN 10 DAYS AFTER INNOCULATION OF 2.5 × 10^6 CELLS I.P.

Treatment	First generation					Second generation				
	No. of animals	Mean wt. gain (gm.)	S.E.*	Mean cell count/$mm.^3$	S.E.*	No. of animals	Mean wt. gain (gm.)	S.E.*	Mean cell count/$mm.^3$	S.E.*
Control group......	10	8.0	±0.61	12.1×10^4	±0.30	10	8.6	±0.46	11.8×10^4	±0.44
20 μc/animal for 5 days i.p..........	10	10.5	±1.90	12.8×10^4	±0.58	10	14.1	±1.52	13.7×10^4	±0.66
15 μc/animal for 5 days i.p..........	10	9.6	±0.82	12.7×10^4	±0.46	10	10.3	±0.73	13.1×10^4	±0.77
10 μc/animal for 5 days i.p..........	10	9.2	±0.67	12.9×10^4	±0.58	10	10.4	±0.60	12.7×10^4	±0.44
2.0 μc/animal for 5 days i.p..........	10	12.5	±1.40	10.9×10^4	±0.38	10	13.6	±1.09	11.3×10^4	±0.33
10 mg. Quinacrine/100 ml. Ascitic Fluid, incubated for 2 hrs. at 37°C .	10	11.1	±0.91	12.2×10^4	±0.41	10	14.7	±0.99	12.9×10^4	±1.44

* S.E. = Standard error of mean.

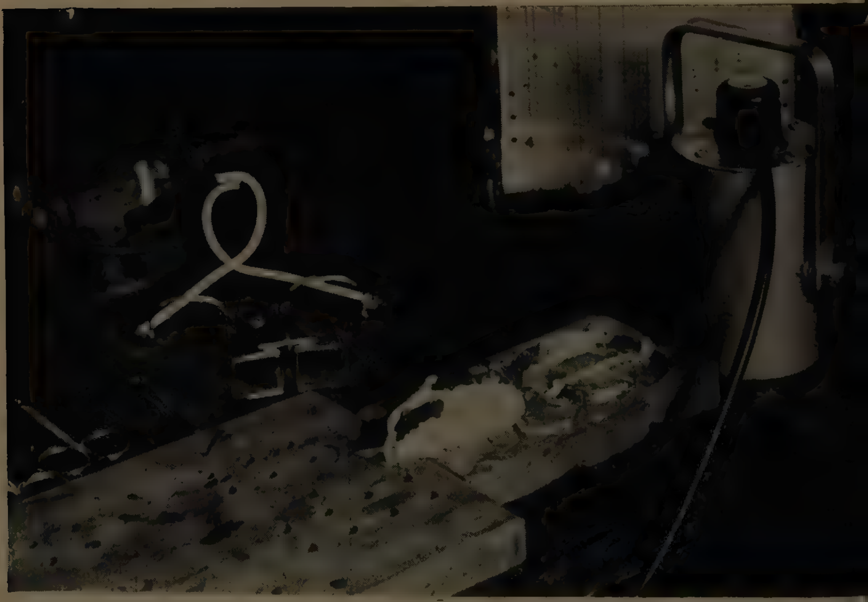


FIGURE 2

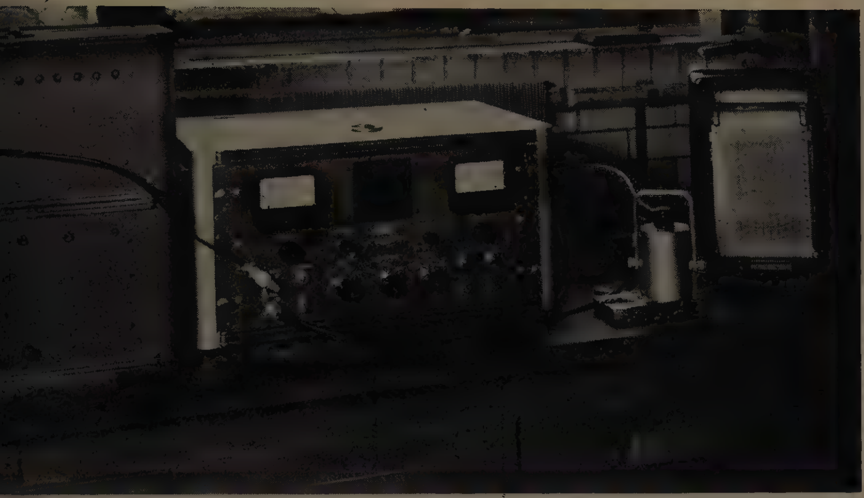


FIGURE 3

nd an Esterline recorder, which continuously graphed radioactivity of the tail FIGURE 3). To test the sensitivity of this method in recording changes in the evel of circulating radioactive particulate matter, various doses of $CrP^{32}O_4$ olloid were injected intravenously. This colloid was rapidly cleared from the lood stream and taken up by the reticuloendothelial system.

It can be seen from FIGURE 4 that it is difficult to distinguish between the 8 nd 10 microcurie dose, but the difference between 8 and 12 microcuries is quite pparent. This method registers the combined radioactive particles in the eins and arteries of the tail. It was found that when blood samples were aken by cutting the tip of the tail, the relative proportion of arterial and venous lood obtained varied with the time of sampling in relation to the time of amutation.

In the experiments to be reported, 1×10^7 washed tumor cells were injected ntravenously. The jugular vein was exposed as it emerged at the cranial part f the pectoral muscles. Pressure was applied to the pectoral muscle overlaying he vein and a 27-guage needle attached to a tuberculin syringe was inserted nto the distended vein through the muscle. After completing the injection, he needle was retracted through the pectoral muscle. Using this procedure, o hemorrhage occurred. Following injection of tumor cells, a number of aninals died with signs of respiratory distress. Since, with the same tumor-cell uspension, some animals died, while others survived, the cause of death lies n the animals rather than in the tumor preparations. Probably individual ifferences in the size and number of pulmonary arteriovenous shunts is inolved.

At various time intervals after the tumor cell injection, the animals were acrificed and bled out. The livers, lungs, spleens, kidneys, adrenals, and tibias ere then removed, weighed, and homogenized with the aid of a Potter-Elve-

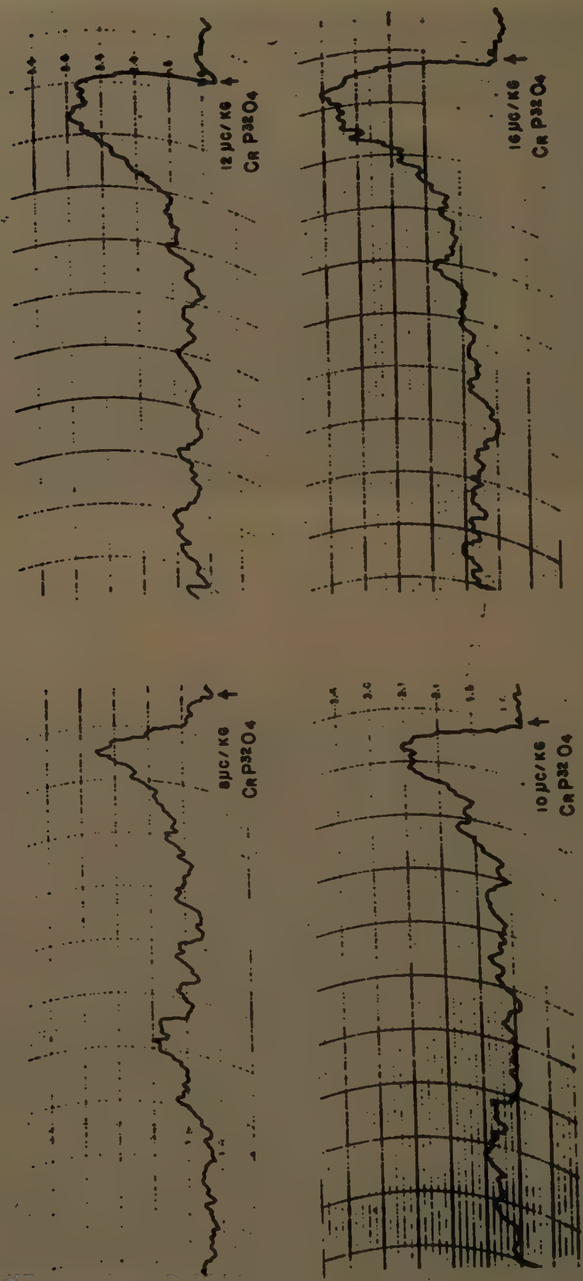
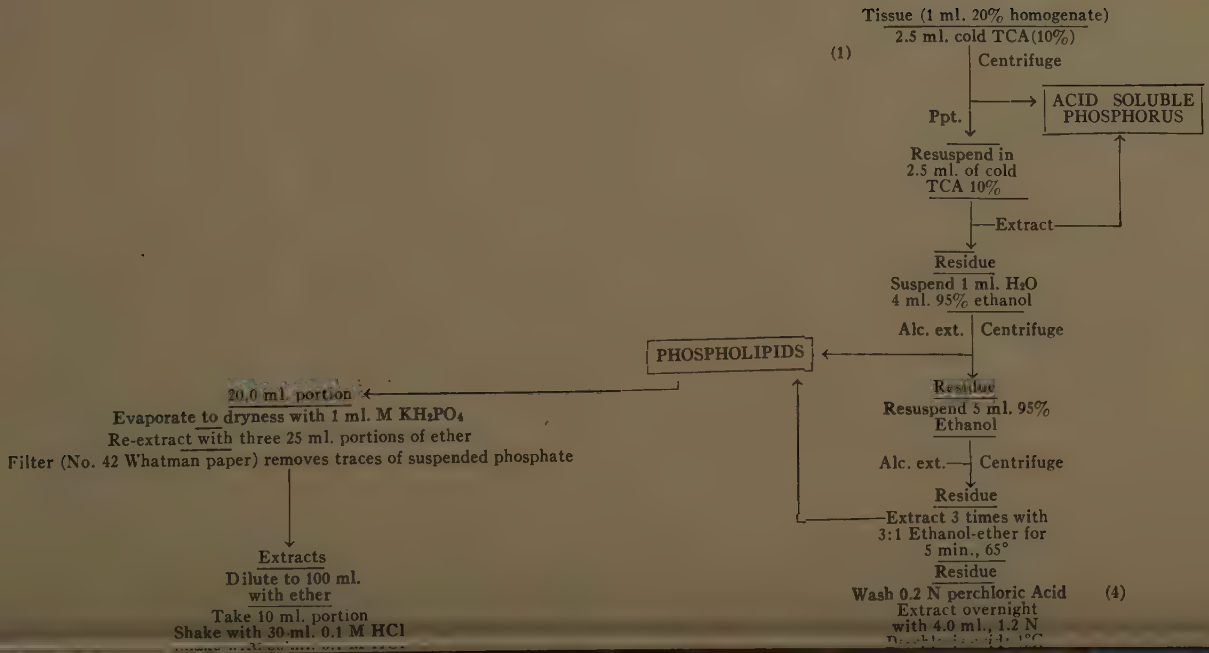


FIGURE 4. Comparison of removal rate of 8, 10, 12, and 16 μc/kg. $CrP^{32}O_4$ from mouse blood (continuou recording method).

jem type homogenizer. The following fractions were prepared from each or- an: (1) organic acid-soluble phosphate; (2) inorganic acid-soluble phosphate; 3) phospholipid; (4) ribonucleic acid; (5) desoxy-ribonucleic acid; and (6) hosphoproteins. The method used is a modification and combination of the ethods of Berenblum and Chain (1938), Schmidt and Thannhauser, Schneider 946), Ogur and Rosen (1950), Ennor and Stocken (1950), Strickland (1952), nd Kit, Bacila, and Barron (1954). FIGURE 5 is a schematic diagram of the actionation procedure. FIGURE 6 outlines the determination of the specific tivity of the inorganic phosphorus fraction. Organic acid-soluble phosphorus action was obtained by retaining the aqueous portion in the above extraction rocedure. Ribonucleic acid, desoxyribonucleic acid, and phosphoprotein frac- ons were freed from contaminating inorganic phosphate as shown in FIGURE 6 norganic acid-soluble phosphorus determination). The aqueous portions ere retained and radioactivity determined. In addition, total phosphorus as determined in all these fractions. Two ml. aliquotes from each fraction ere pipetted on metal planchets. One drop of Duponol solution was added each to facilitate even drying. Samples were dried in an incubator at 37° C. r 24 hours and were counted in a gas-flow counter. Results are expressed as ounts/minute/gram organ, except for the adrenal gland, the valves of which ere expressed as counts/minute/whole organ and blood, which was calculated counts/minute/milliliter. In each experiment, a sample of the tumor-cell ispension used for injection was fractionated as described above. The total umber of cells/cubic millimeter was counted in a hemacytometer. Thus, the arious biochemical fractions could be expressed per injected tumor cell. From e specific activity of the above fractions in the various organs after injection f labeled tumor cells and the specific activity of individual tumor cells, the ossible maximal number of "biochemically intact" tumor cells could be established in each organ. After theoretically assembling the possible maximal umber of tumor cells, quantities of the various components remaining unaccounted for were regarded as originating from disintegrated tumor cells. Of ourse, in all these studies, exchange of P^{32} between labeled, washed tumor cells nd tissues of the host represents a serious source of error. Obviously, these ethods are applicable only to short term experiments. The closer the readgs are taken to the injection of tumor-cell suspensions, the more reliable are e results.

We attempted to develop a second method for detecting the distribution of mbolic tumor cells, using nonradioactive markers, and compared the results f the two methods. We found that 10 mg. of quinacrine added to 100 ml. of scites and incubated for two hours at 37° C. will be taken up by the tumor ells and, if present, by the leukocytes and macrophages in the ascites. Labeled ells will exhibit fluorescence under the ultraviolet microscope. In each field, e total number of cells was counted, then the ascites was switched to the ulraviolet light source and the relative concentration of fluorescent cells was stablished. If labeled ascites was centrifuged and the supernate mixed with onlabeled ascites tumor cells, no fluorescence of the latter was evidenced, inicating that free quinacrine concentration in the ascites fluid is too low to be


Tissue (1 ml. 20% homogenate)
2.5 ml. cold TCA (10%)
(1)
Centrifuge
Ppt.
ACID SOLUBLE PHOSPHORUS
Resuspend in 2.5 ml. of cold TCA 10%
Extract
Residue
Suspend 1 ml. H2O 4 ml. 95% ethanol
Alc. ext.
Centrifuge
PHOSPHOLIPIDS
Residue
Resuspend 5 ml. 95% Ethanol
Alc. ext.
Centrifuge
Residue
Extract 3 times with 3:1 Ethanol-ether for 5 min., 65°
Residue
Wash 0.2 N perchloric Acid
(4)
Extract overnight with 4.0 ml., 1.2 N
20.0 ml. portion
Evaporate to dryness with 1 ml. M KH2PO4
Re-extract with three 25 ml. portions of ether
Filter (No. 42 Whatman paper) removes traces of suspended phosphate
Extracts
Dilute to 100 ml. with ether
Take 10 ml. portion
Shake with 30 ml. 0.1 M HCl

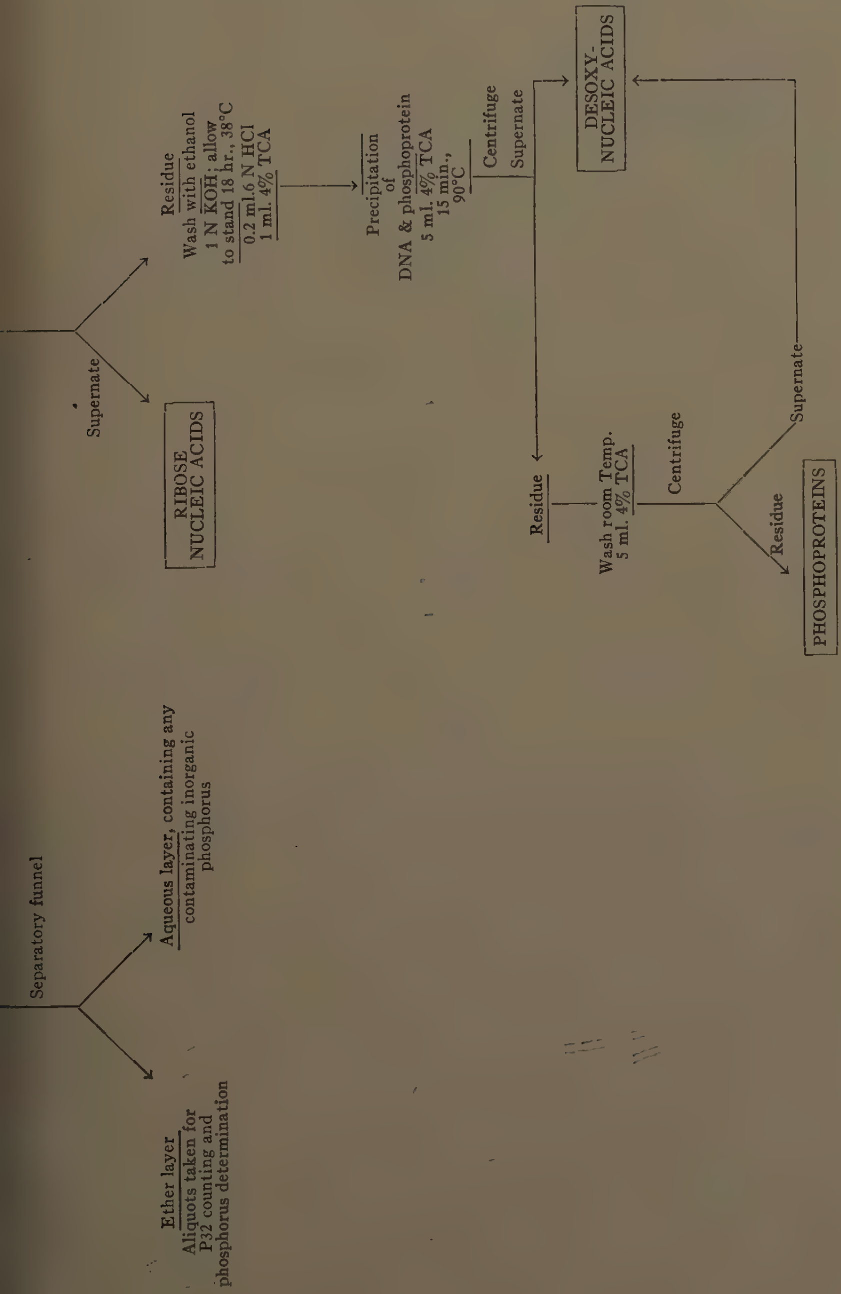

Separatory funnel
Ether layer
Aliquots taken for P32 counting and phosphorus determination
Aqueous layer, containing any contaminating inorganic phosphorus
Supernate
RIBOSE NUCLEIC ACIDS
Residue
Wash with ethanol
1 N KOH; allow to stand 18 hr., 38°C
0.2 ml. 6 N HCl
1 ml. 4% TCA
Precipitation of DNA & phosphoprotein
5 ml. 4% TCA
15 min., 90°C
Centrifuge
Supernate
DESOXY-NUCLEIC ACIDS
Residue
Wash room Temp.
5 ml. 4% TCA
Centrifuge
Supernate
Residue
PHOSPHOPROTEINS

FIGURE 5

Acid soluble phosphorus fraction:

Determination of inorganic phosphorus (2), (5)

Reagents

1. 10N H_2SO_4
2. N H_2SO_4 (reagent 1 diluted 10 times)
3. Five per cent ammonium molybdate
4. $SnCl_2$ stock solution: 10 gm. $SnCl_2$/25 ml. Conc. HCl
5. Solution No. 4 diluted 200 times with N H_2SO_4 (always freshly prepared).
6. Isobutanol
7. Ethanol
8. NaCl 5%

Procedure

0.5–2.5 ml. Aliquot of acid soluble fraction
2.5 ml. Ammonium molybdate 5%
0.5 ml. 10N H_2SO_4
2.0 ml. NaCl 5%
Sufficient distilled water to make 10 cc.
20° C.

Allow 15 second reaction time

Add 20.0 ml. isobutanol & shake for 10 seconds

Remove aqueous layer, shake isobutanol layer twice with 10
portions in H_2SO_4

Aliquot may be used for determining total inorganic phosphorus colorimetrically.

Shake isobutanol layer 30 sec. with a 30 ml. portion of the dilut
$SnCl_2$ solution

Alcohol layer retained and P^{32} counts made after making up
25 ml. with ethanol

FIGURE 6

able to label further cells. TABLE 2 shows that the growth potentials of qui acrine-labeled ascites tumors do not differ significantly from that of the co trols.

In the present report, only results obtained with P^{32}-labeled Ehrlich ascit carcinoma will be reported, since sufficient data are as yet unavailable on e periments in which other tumors or methods were used.

Results

Transpulmonary passage of tumor cell emboli in various strains of mice. Wi (1952), Lachman (1954), and Zeidman and Buss (1952) reviewed the literatu on the transpulmonary passage of tumor-cell emboli. The latter authors i jected tumor-cell suspensions intravenously and simultaneously collected aort blood. Aortic blood was then injected intravenously into a recipient anima Tumors developed in the second animal, indicating that embolic cells pass immediately through the lungs of the first animal. In our experiments, tumo cell suspensions were injected into the jugular vein, and radioactivity w recorded simultaneously over the tail. FIGURE 7 shows such a recording in Swiss mouse. Immediate transpulmonary passage and repeated circulati of labeled tumor cells through the lungs is evident. In all of these experimen 1×10^7 tumor cells were injected. While large individual variations we noted, the impression was gained that transpulmonary passage is less effecti in $C_{57}Bl/6$, DBA/2, and Strong A animals than in Webster Swiss or C_3H mic FIGURE 8 shows sections from a recording in a Strong A mouse. In this e periment, very few embolic cells passed the pulmonary barrier immediatel On the other hand, when the Geiger counter was placed over the lungs, hig activity was recorded. This indicates that most cells are lodged in the lung After a relatively short period of time, it seems that some cells are released fro

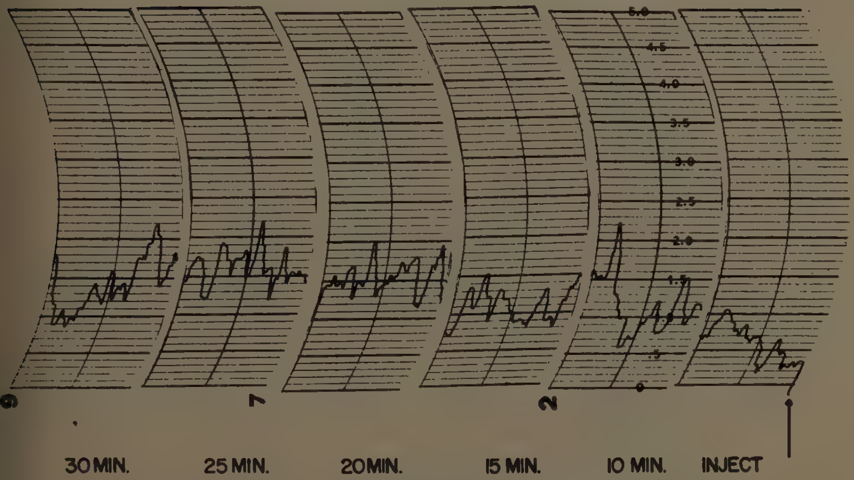


FIGURE 7. Swiss mouse injected with 10^7 Ehrlich cells.

he lungs into the systemic circulation. Secondary release was observed only ccasionally, although most animals were followed up for several hours. FIGURE 9 shows a recording in a C_{57}Bl/6 mouse after intravenous injection of × 10^7 Ehrlich cells. The strain difference in immediate pulmonary passage f embolic tumor cells may be due to differences in the number and size of pulnonary arteriovenous shunts.

Role of Blood Clotting in the Distribution of Embolic Tumor Cells

Lawrence, Moore, and Bernstein (1953) observed that suspensions of V_2 arcinoma injected intravenously to rabbits caused a large percentage of acute leath due to thromboembolism in the pulmonary system and in the right heart. The tumor itself exhibited high thromboplastic activity. When the animals vere heparinized prior to injection of tumor cells, fewer deaths occurred. In he survivors, more animals exhibited metastasis beyond the pulmonary barrier, nd a few animals had no pulmonary tumor. Zeidman and Buss (1952) found o difference in the metastatic pattern whether the tumor cell suspension was njected in a dilute-heparin solution or not. In our experiments, mice were eparinized with 10 mg. heparin sodium per mouse 10 minutes prior to injection f 1 × 10^7 Ehrlich cells. Large individual variations were noticed, but the irculating level of radioactivity was consistently higher than in the controls. FIGURE 10 shows the records of such an experiment. The level of peripheral adioactivity depends on the rate of passage of labeled cells through the pulmonary barrier and on the rate of removal of embolic cells by lodgment in the arious organs. This relationship can be judged best from the degree of radioactivity recorded immediately after the injection of tumor cells. Rate of disappearance from the blood stream is not considered a good criterion, since embolic tumor cells seem to circulate for many hours in the blood stream. In this ime, exchange of P^{32} between tumor cells and normal tissues may represent a erious source of error.

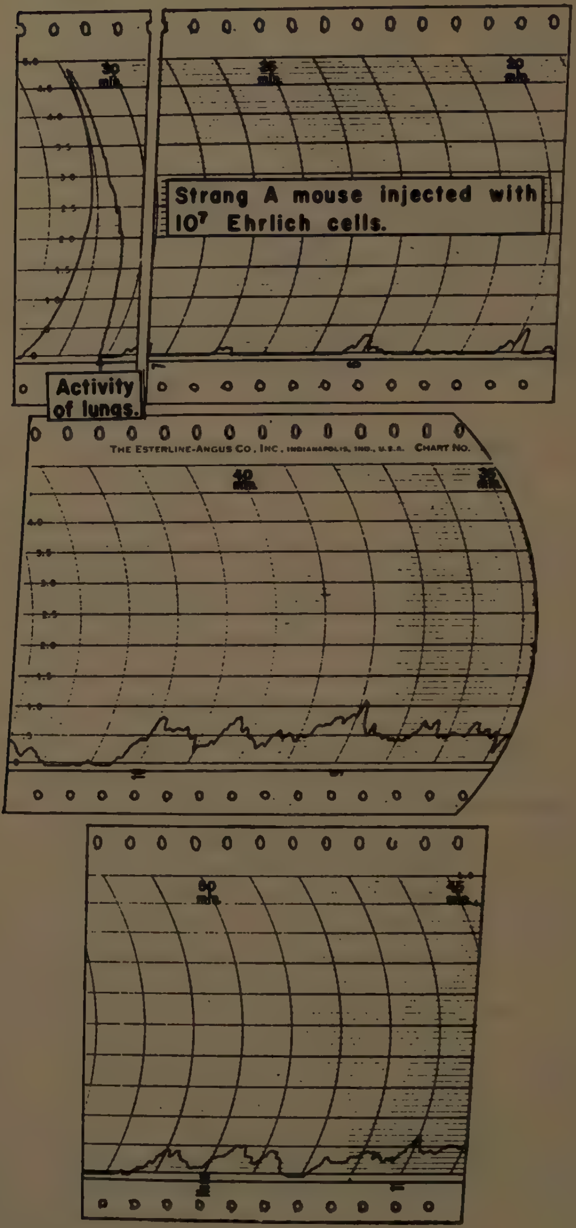

30 min.
25 min.
20 min.
Strong A mouse injected with 10^7 Ehrlich cells.
Activity of lungs.
THE ESTERLINE-ANGUS CO., INC., INDIANAPOLIS, IND., U.S.A. CHART NO.
40 min.
35 min.
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
50 min.
45 min.

FIGURE 8

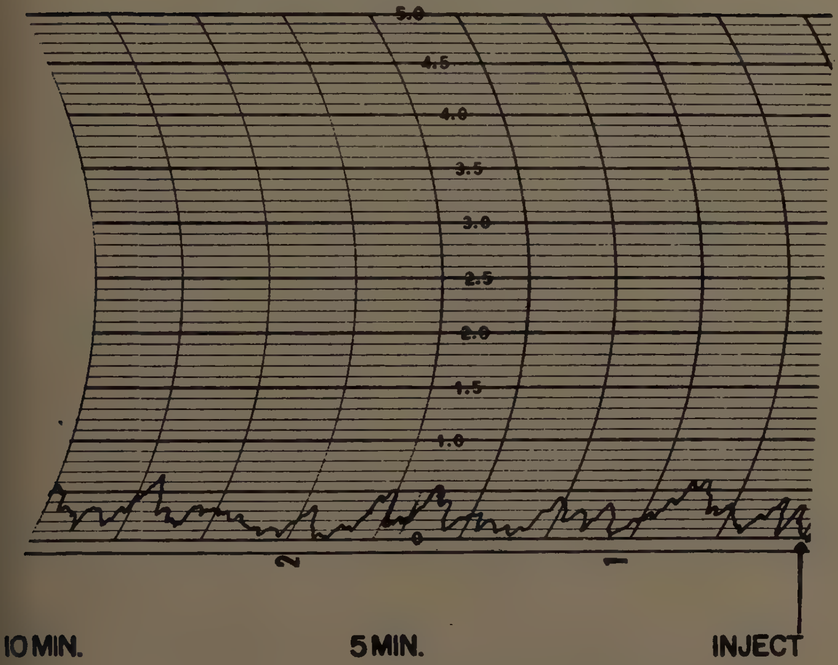


FIGURE 9. C_{57}Bl/6 mouse injected with 10^7 Ehrlich cells.

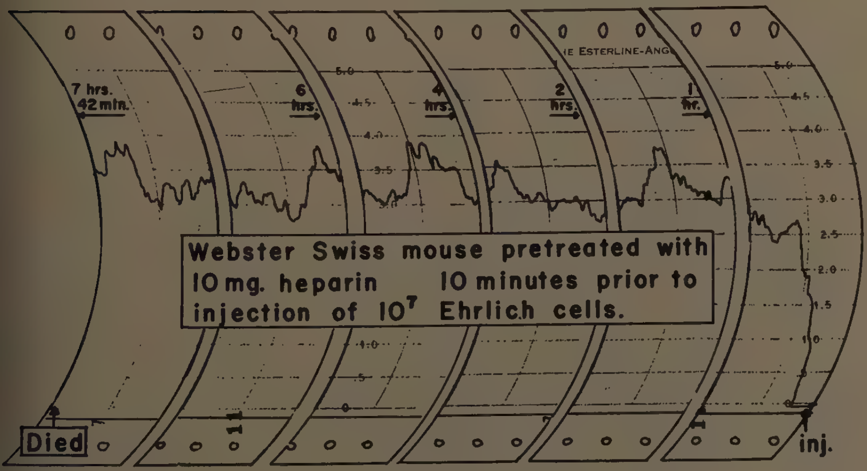


FIGURE 10

Effect of Thorotrast on Tumor-Cell Embolism

Thorotrast (colloidal thorium dioxide) is known to be taken up by the reticuloendothelial system and inhibit its antibody-forming (Maher 1944, Lewis 1954) and phagocytic (Martin, Kerby, and Holland 1952) activity. Bernard, Dutton, and Kadakovich (1955) found that Thorotrast did not increase the

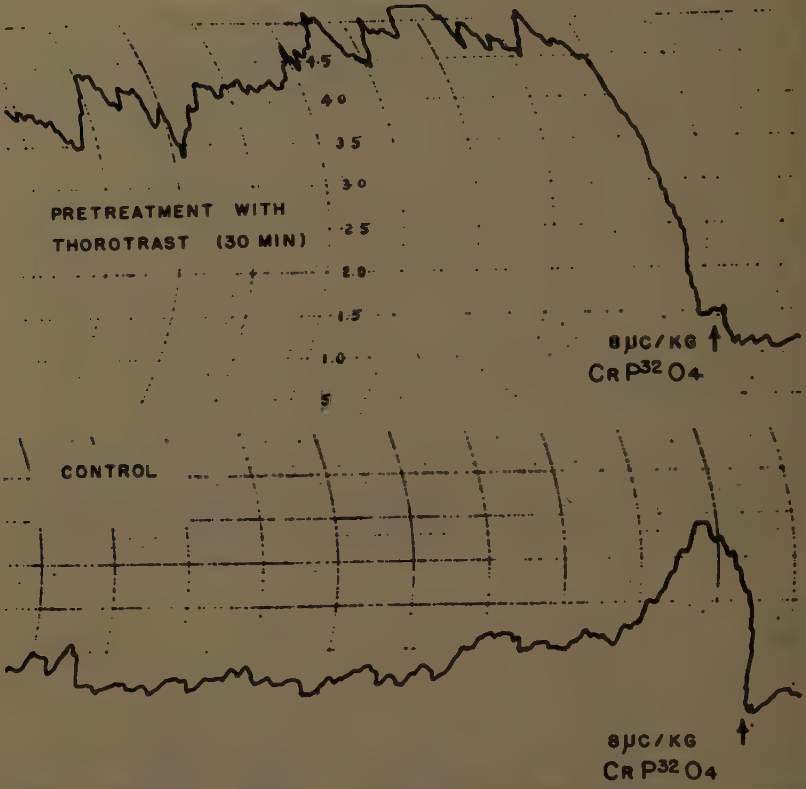


FIGURE 11. Effect of 0.5 ml. Thorotrast on removal rate of 8 μc/kg. $CrP^{32}O_4$ from mouse blood (continuou recording method).

takes of transplanted tumors nor did it alter induced immunologic resistance t transplanted tumors.

In preliminary experiments, we have compared the disappearance of colloida $Cr^{P^{32}}O_4$ from the blood stream of normal Swiss mice and animals that wer pretreated with 0.5 ml./mouse of intravenous Thorotrast. Disappearance o the colloid was always greatly reduced by Thorotrast pretreatment (FIGURE 11) Similarly, 0.5 ml. of Thorotrast given intravenously 24 hours prior to injectio of Ehrlich ascites tumor cells greatly increased the level of circulating emboli cells. Such an experiment is shown in FIGURE 12. In this instance, upon injection of the labeled cells, the indicator went off the chart and it was necessar to change to a 10-times reduction. This phenomenon seems to be difficult t explain. It is unlikely that the removal of embolic tumor cells from the circulation depends on phagocytosis by the reticuloendothelial system.

Susceptibility and Metastatic Distribution of the Ehrlich Ascites Tumor in Variou Strains of Mice

The Ehrlich ascites carcinoma is known to grow in a variety of mouse strains and is a suitable subject to study the role of genetic factors in strain differenc

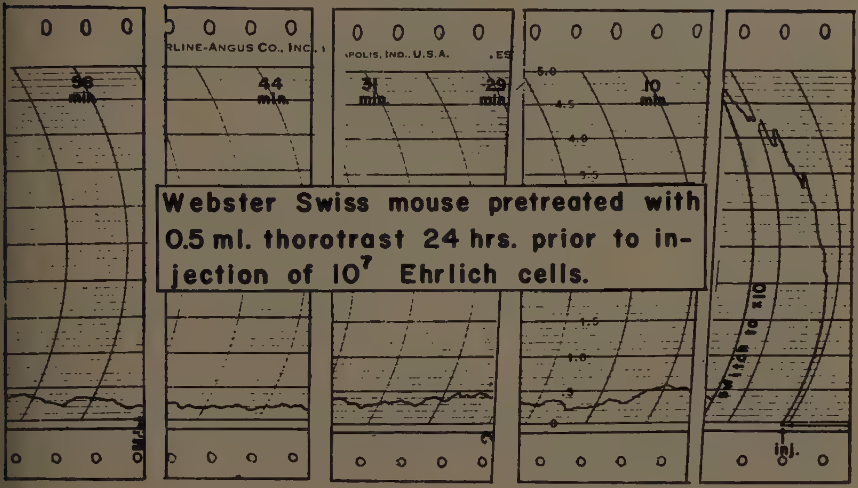


FIGURE 12

in susceptibility to its metastatic spread. As an initial approach, subcutaneous and intraperitoneal growth rates were determined in various strains and compared with initial metastatic distribution (using labeled tumor-cell emboli) and development of metastatic tumors. FIGURE 13 shows the weight of subcutaneous tumors in six strains 28 days after inoculation of one million tumor cells, and also mortality curves for the same strains following intraperitoneal injection of the same number of cells. It appears that the AK and Strong A mice are the most susceptible and the $C_{57}Bl/6$ mice the most resistant, while the other strains occupy an intermediate position. Development of metastases was determined following intravenous inoculation by sacrificing the animals as soon as they appeared to be moribund, and counting the number of macroscopically evident metastases during autopsy. Thus, the end point in this series varied from animal to animal. Within the time interval in which the animals became moribund, macroscopic metastases were found only in the lungs and liver. It appears from TABLE 3 that the occurrence of metastases in the various strains showed some correlation with the subcutaneous and intraperitoneal growth rates in these same strains.

TABLE 4 shows the distribution of the various P^{32} containing biochemical fractions in organs and blood of several strains of mice at various time intervals following intravenous injection. From the content of the same fractions in the inoculum and the inoculum cell count, the content of one tumor cell was calculated. From the occurrence of the various fractions in the organs, the maximal possible number of intact cells was calculated and indicated in the table. It is apparent that, after even a very brief period of time, a large number of tumor cells disintegrate and their P^{32}-containing fractions are exchanged between organs. On the basis of the small number of experiments available and the large degree of their individual variation, no difference can be postulated between

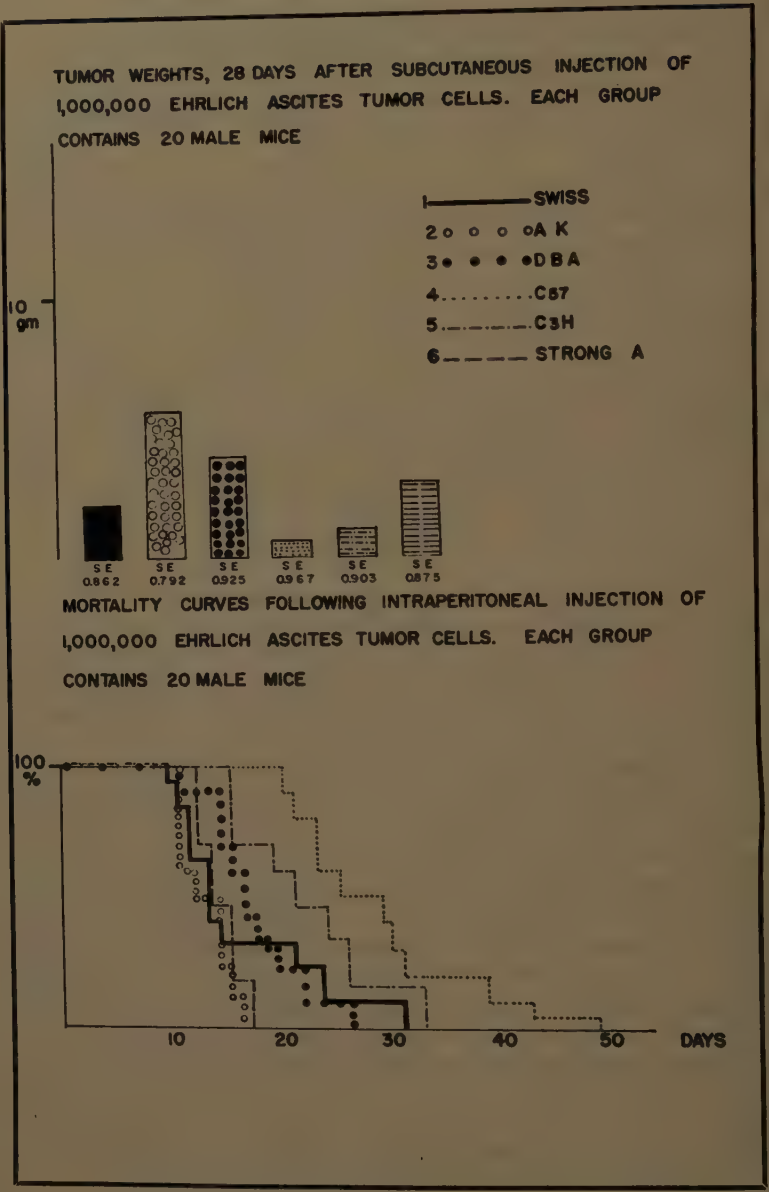


FIGURE 13

initial distribution of embolic tumor cells in the strains observed. The apparen differences in the development of metastases between these strains seem to b based on differences in survival rate of embolic tumor cells rather than on differences in initial distribution.

TABLE 3

METASTASES IN MALE MICE AFTER INJECTION OF 2 × 10^7 EHRLICH ASCITES TUMOR CELLS INTO THE TAIL VEIN*

0 = no metastasis
1 = 1 metastasis
2 = 2 to 10 metastases
3 = more than 10 metastases

Mouse strain	No. injected	Lungs				Liver			
		0	1	2	3	0	1	2	3
Swiss	20	0	3	11	6	2	9	7	2
Strong A	20	0	6	10	4	3	7	4	6
C_{57}Bl/6	20	3	12	5	1	8	7	5	0
DBA/2	20	0	9	8	3	4	14	2	0

* Animals were sacrificed individually when they appeared to be moribund.

Thus, the differences in metastic patterns between mouse strains are more likely to be due to immunogenetic and nutritional factors than to anatomical or hemodynamic differences. It may be argued that immunogenetic "soil" factors may be of importance only when the tumor and host are not completely homologous genetically. It should be mentioned that most mouse and rat tumors used in the study of metastasis fall in the same category.

TABLE 5 shows the initial distribution of embolic tumor cells in Swiss mice. Ehrlich ascites carcinoma was implanted subcutaneously into the tails of these mice two weeks prior to the experiment. After 10 days of growth, the portion of the tail carrying the subcutaneous tumor was amputated. It is apparent that embolic tumor cells rapidly disintegrate in the organs of these preimmunized animals.

Summary

(1) A method of recording continuously the appearance, circulation, and disappearance of P^{32}-labeled embolic tumor cells in the blood stream has been described.

(2) The possibility of direct passage and repeated circulation of tumor cells through the lungs has been demonstrated.

(3) Biochemical fractions containing P^{32} were isolated from the organs of the recipient. On the basis of similar fractionation performed with a sample from the injected tumor cells, the maximal possible number of intact tumor cells in the organs could be calculated.

(4) Differences were found in the subcutaneous and intraperitoneal growth rate and in the development of metastases of the Ehrlich ascites carcinoma between various strains of mice. No differences were found, however, concerning the initial distribution of embolic tumor cells. Thus differences in the development of metastases between these strains seem to be due to immnuogenetic and/or nutritional factors.

(5) In preimmunized animals, rapid disintegration of embolic tumor cells could be noticed.

Table 4

Fractionation Studies in Mice Injected with 1×10^7-Ehrlich Ascites Tumor Cells into the Jugular Vein

Mouse strain	Organ	Fraction***	Five minutes		Thirty minutes		Two hours		Six hours	
			Counts/min./* gm. organ	Poss. max.** No. intact cells	Counts/min./* gm. organ	Poss. max.** No. intact cells	Counts/min./* gm. organ	Poss. max.** No. intact cells	Counts/min.* gm. organ	Poss. max.** No. intact cells
A/Jax	Liver	OASP	124,314	2.1×10^6	5,836	1.9×10^5	8,750	3.5×10^6	1,008	2.4×10^6
		IASP	1,083		580		3,750		6,336	
		PL	18,808		8,928		6,433		22,598	
		RNA	540		369		4,170		2,395	
		DNA	6,325		1,186		220		112	
		PP	33,330		1,354		2,200		1,224	
	Lungs	OASP	58,563	8.5×10^6	12,483	2.9×10^5	5,720	9.5×10^5	1,092	6.0×10^5
		IASP	1,671		2,137		1,716		728	
		PL	60,500		17,850		19,104		10,640	
		RNA	2,214		2,856		6,840		605	
		DNA	1,069		3,320		765		305	
		PP	1,001,330		28,800		3,833		2,501	
	Spleen	OASP	226,582	4.9×10^6	95,139	2.8×10^6	4,576	1.1×10^6	2,004	6.7×10^5
		IASP	1,100		4,633		10,868		4,008	
		PL	34,722		13,700		3,581		7,463	
		RNA	6,128		4,045		1,140		666	
		DNA	1,663		5,440		864		336	
		PP	485,980		27,450		3,264		3,808	
	Kidney	OASP	278,070	1.4×10^7	4,724	1.7×10^6	800	6.0×10^5	444	7.4×10^5
		IASP	7,453		1,427		9,200		1,332	
		PL	24,206		10,790		1,062		3,327	
		RNA	46,777		4,499		798		740	
		DNA	990		2,561		536		148	
		PP	3,610,178		17,138		2,546		3,085	
	Bone (Tibia)	OASP	125,000	1.5×10^5	111,150	3.4×10^6	—	—	2,000	1.9×10^6
		IASP	9,370		4,800		—		12,118	
		PL	61,200		11,942		2,004		7,515	
		RNA	25,890		11,424		1,064		1,995	
		DNA	5,170		7,712		—		335	
		PP	314,452		32,840		8,040		11,055	
	Blood	OASP	1,048	3.2×10^7	1,197	1.5×10^6	240	6.1×10^5	240	2.8×10^4
		IASP	1,029		249		800		240	
		PL	1,558		1,342		1,269		50	
		RNA	858		1,426		612		80	
		DNA	504		326		202		35	
		PP	3,333		14,154		777		387	
	Adrenals	OASP	1,812	4.5×10^5	378	6.0×10^5	—	—	80	4×10^4
		IASP	122		152		200		160	
		PL	499		685		84		167	

Mouse strain	Organ	Fraction***	Counts/ min./*gm. organ	Poss. max.** No. intact cells	Counts/ min./*gm. organ	Poss. max.** No. intact cells
Webster Swiss	Liver	OASP	1,926	3.1 × 10⁵	950	7.6 × 10⁴
		IASP	15,408		38,016	
		PL	6,220		990	
		RNA	9,541		1,141	
		DNA	5,685		4,510	
		PP	9,329		103,122	
	Lungs	OASP	5,040	3.2 × 10⁶	750	2.6 × 10⁵
		IASP	39,060		6,000	
		PL	234,675		3,432	
		RNA	176,300		5,632	
		DNA	200,980		3,168	
		PP	38,587		52,400	
	Spleen	OASP	1,368	5.4 × 10⁴	—	—
		IASP	8,208		14,007	
		PL	2,565		684	
		RNA	702		684	
		DNA	12,010		528	
		PP	6,600		15,600	
	Kidney	OASP	1,044	6.5 × 10⁵	924	4.9 × 10⁴
		IASP	49,068		4,620	
		PL	49,068		2,310	
		RNA	21,420		632	
		DNA	12,261		800	
		PP	12,725		17,596	
	Bone (Tibia)	OASP	—	—	—	—
		IASP	2,640		—	
		PL	18,700		7,150	
		RNA	12,628		3,516	
		DNA	4,788		5,880	
		PP	61,492		48,216	
	Blood	OASP	6,240	3.1 × 10⁵	960	1.8 × 10⁵
		IASP	77,760		12,500	
		PL	4,000		3,600	
		RNA	16,400		2,296	
		DNA	33,264		1,804	
		PP	5,988		7,560	
	Adrenals	OASP	—	—	—	—
		IASP	2,568		880	
		PL	—		150	
		RNA	1,025		56,956	
		DNA	714		105	
		PP	2,100		1,071	

Mouse strain	Origin	Fraction***	Counts/ min./* gm. organ	Poss. max.** No. intact cells	Counts/ min./* gm. organ	Poss. max.** No. intact cells
$C_{57}Bl/6$	Liver	OASP	3,117	3.7 × 10⁵	874	1.4 × 10⁶
		IASP	489		1,464	
		PL	447		4,050	
		RNA	217		1,183	
		DNA	874		4,462	
		PP	3,764		25,930	
	Lungs	OASP	4,972	2.6 × 10⁶	41,080	2.6 × 10⁶
		IASP	7,417		3,314	
		PL	46,035		4,050	
		RNA	12,500		1,180	
		DNA	3,400		4,462	
		PP	26,550		25,930	
	Spleen	OASP	27,536	1.1 × 10⁵	6,700	3.6 × 10⁶
		IASP	5,250		11,117	
		PL	6,665		1,150	
		RNA	28,660		1,785	
		DNA	3,543		8,340	
		PP	52,305		71,925	
	Kidney	OASP	3,920	6.3 × 10⁶	9,200	3.5 × 10⁵
		IASP	1,241		8,428	
		PL	2,177		3,386	
		RNA	5,193		2,331	
		DNA	2,435		3,346	
		PP	42,810		29,722	
	Bone (Tibia)	OASP	26,150	1.7 × 10⁷	10,935	3.8 × 10⁶
		IASP	40,250		2,999	
		PL	14,700		865	
		RNA	87,250		23,250	
		DNA	11,900		4,220	
		PP	116,512		38,825	
	Blood	OASP	304	4.9 × 10⁵	493	3.5 × 10⁵
		IASP	795		539	
		PL	336		544	
		RNA	4,046		3,458	
		DNA	794		518	
		PP	36,064		7,750	
	Adrenals	OASP	362	1.6 × 10⁵	840	2.1 × 10⁵
		IASP	507		915	
		PL	91		78	
		RNA	405		20,430	
		DNA	114		2,018	
		PP	1,572		1,470	

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* Calculated to counts per minute gm. organ except adrenals (calculated as cells per whole organ) and blood (calculated per ml.).

** Cells expressed as cells per gm. of organ except adrenals (calculated as cells per whole organ) and blood (calculated per ml.).

*** *Abbreviations:* OASP—Organic acid-soluble phosphorus; IASP—Inorganic acid-soluble phosphorus; PL—Phospholipids; RNA—Ribosenucleic acid; DNA—Desoxyribosenucleic acid; PP—Phosphoproteins.

TABLE 5

FRACTIONATION STUDIES IN MICE INJECTED WITH 1 × 10^7-EHRLICH ASCITES TUMOR CELLS INTO THE JUGULAR VEIN

Immune Webster Swiss

Organ	Fraction***	Five minutes		Thirty minutes	
		Counts/min.* gm. organ	Poss. max.** No. intact cells	Counts/min./* gm. organ.	Poss. max.** No. intact cells
Liver	OASP	840	7.6 × 10^5	1,512	2.2 × 10^5
	IASP	5,880		23,058	
	PL	5,640		4,185	
	RNA	10,800		3,060	
	DNA	24,756		27,900	
	PP	16,032		23,120	
Lungs	OASP	1,078	4.6 × 10^6	2,324	6.1 × 10^5
	IASP	37,730		17,430	
	PL	89,320		22,825	
	RNA	66,528		8,632	
	DNA	39,886		202,188	
	PP	55,132		10,120	
Spleen	OASP	—	—	—	—
	IASP	4,212		9,576	
	PL	2,695		950	
	RNA	4,260		1,824	
	DNA	68,376		11,217	
	PP	65,989		41,040	
Kidney	OASP	—	—	—	
	IASP	74,920		36,036	—
	PL	3,960		4,290	
	RNA	3,408		7,436	
	DNA	44,880		42,775	
	PP	108,108		91,520	
Bone	OASP	—	—	—	—
	IASP	—		52,052	
	PL	—		—	
	RNA	—		69,598	
	DNA	16,950		31,303	
	PP	26,130		130,240	
Blood	OASP	2,100	2.1 × 10^5	1,664	1.6 × 10^6
	IASP	112,840		35,938	
	PL	15,800		35,870	
	RNA	2,920		23,120	
	DNA	40,900		30,328	
	PP	49,520		136,000	
Adrenals	OASP	—	—	—	
	IASP	6,160		5,180	—
	PL	500		1,700	
	RNA	160		4,400	
	DNA	2,080		12,200	
	PP	3,480		5,680	
		Table 5 (A)		Table 5 (B)	

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* Calculated to counts per min. gm. organ except adrenals (calculated as cells per whole organ) and blo (calculated per ml.).

** Cells expressed as cells per gm. of organ except adrenals (calculated as cells per whole organ) and blo (calculated per ml.).

*** Abbreviations: OASP—Organic acid-soluble phosphorus; IASP—Inorganic acid-soluble phosphoru PL—Phospholipids; RNA—Ribosenucleic acid; DNA—Desoxyribosenucleic acid; PP—Phosphoproteins.

(6) Heparin and Thorotrast seemed to accelerate the pulmonary passage of umor cells, or else to delay their fixation in organs. The effect of heparin is robably related to the role of blood clotting in the lodgment of tumor-cell mboli. It appears unlikely that the blocking effect of Thorotrast on the eticuloendothelial system is responsible for its effect.

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Discussion of the Paper

Doctor Irving Zeidman (*Pathology Department, University of Pennsylvani School of Medicine, Philadelphia, Pa*): Doctor Ambrus is to be congratulate for his very informative presentation dealing with important problems concerning tumor-cell embolism. One very obvious conclusion from his work i that some tumor cells pass immediately through the lung circulation. Th problem of immediate transpulmonary passage of tumor-cell emboli intereste me several years ago, and I should like to discuss an experiment that correlate well with the work of Doctor Ambrus. This experiment was performed to determine whether or not tumor-cell emboli could pass immediately through th lungs. Transplantable tumors were used in rabbits and rats. A tumor suspension was injected intravenously and, simultaneously, the aortic blood wa collected. This aortic blood was then injected intravenously into a secon normal animal. If the second animal developed tumors, this would mean, ther that tumor cells passed immediately through the pulmonary circulation of th first animal. Immediate transpulmonary passage of tumor cells occurred wit all tumors in rabbits and rats. Now the incidence of such passage differe The Brown-Pearce tumor cells revealed a significantly higher incidence of immediate transpulmonary passage than did the V_2 carcinoma. Both tumor were in rabbits. With both tumors, the same number of tumor cells were use for the initial intravenous injection. How can we explain this difference i incidence of transpulmonary passage? Conceivably, cell size may play a role— so the sizes of the tumor cells were measured. The Brown-Pearce cell was little larger, if anything, than the V_2 carcinoma cell. Obviously, cell size i not the important factor. Also, the ascites cells are really enormous compare to the normal cells of the mouse. Yet these cells, as Doctor Ambrus has shown probably pass repeatedly through the lung circulation before being stopped. One point requiring further investigation is the rigidity of the cells. V_2 cells may be rigid and stick to the capillary walls, whereas Brown-Pearce cells may travel through the capillaries by elongating like an oil droplet. Certainly our concepts concerning arrest of tumor-cell emboli need some modification. Emboli coming from solid local tumors may circulate and recirculate for some time before lodging.

Another aspect of Doctor Ambrus's work to be discussed concerns the action of heparin and, particularly, of Thorotrast. These agents delay the decline of the tail count. I cannot offer any concrete explanation for this phenomenon, but perhaps I can suggest some reasons that could be checked. It may be that these agents lead to a more extensive destruction of tumor cells, with subsequent release of the radioactivity free in the blood stream; or these agents may produce a pulmonary vasodilation that permits a longer interval of passage through the lungs and other organs. Still another possibility is this. Many radioactive tumor cells spontaneously disintegrate in the blood stream and release radioactive particulate matter. This matter is normally phagocytized by cells of the reticuloendothelial system. Thorotrast would decrease this phagocytosis by the well-known mechanism of reticuloendothelial blockage. Heparin could conceivably decrease the phagocytosis by interfering with a surface coating mechanism necessary for phagocytosis. With both drugs, then, there would be decreased phagocytosis of radioactive particulates. The latter would circulate in the blood stream, thereby delaying the decline of the tail count.

The last point to be considered is the determination of the rate of disintegration of tumor cells by the method of cell fractionation and localization of radioactive phosphorus in different constituents. The reliability of this method depends on a number of presumptions that are open to question. I should like to suggest another method that checks on the results obtained by Doctor Ambrus. This other method depends simply on the recognition of ascites tumor cells in routine histological sections of the tumors. It so happens that these tumor cells are so large that they do not resemble other normal cells of the mouse. Ascites cells can be recognized and counted in tissue sections. A few years ago, I injected ascites tumor cells intravenously into mice, and sacrificed the mice immediately afterwards. Then microscopic sections of various organs were examined. The large ascites cells were found in the lung, as expected. But some tumor cells went through the lungs immediately, for large numbers were seen in the liver and kidney. The ascites cell nuclei are considerably larger than normal nuclei and so are readily identifiable. Therefore, one may apply this method to Doctor Ambrus's problem on finding what happens to these tumor cells. One can do this simply by ascites cell counts on routine sections of different organs.

MERITS OF ASCITES TUMORS FOR CHEMOTHERAPEUTIC SCREENING

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PART I*

By Kanematsu Sugiura

In recent years, much interest has developed in the use of ascites tumors i experimental cancer research, especially in cytology and biochemistry of tumo cells. The ascites tumors, however, have been used comparatively infrequentl by investigators for chemotherapeutic studies.[1] The ascites tumors provid an easy challenge to chemotherapeutic agents, because the intraperitoneal injection of compounds against the intraperitoneal ascites form of tumors is, in effect, an *in vivo-in vitro* test. It is thus possible to obtain an idea of the action of a compound without as great a degree of intervention by other organs.

Materials and Methods

The Ehrlich ascites carcinoma, Krebs 2 ascites carcinoma, and Sarcoma 18 ascites tumor were chosen for the present study.

Upon intraperitoneal injection of fresh ascites fluid containing about on million cancer cells (FIGURE 1), mice regularly develop large amounts of milk ascites (5 to 20 cc.) in 7 to 14 days (FIGURE 2) and die in 7 to 20 days. Th tumors have 100 per cent takes and, generally, no spontaneous regressions.

The milky fluid of Ehrlich ascites tumor contains about 25 million cance cells/cc., while Krebs 2 and Sarcoma 180 ascites tumors contain about 10 million cancer cells/cc. These peritoneal exudates contain 5 to 10 per cen of normal cells, such as leukocytes, histiocytes, and mesothelial cells.

There are several ways of determining the effectiveness of a compound agains the ascites form of tumors: (1) cytological examination of ascites tumor cell from treated animals;[2, 3, 4] (2) counting the number of tumor cells at variou time intervals in ascitic fluid after treatment;[5] (3) determination of the ce volume obtained by means of centrifugation of aliquots of ascitic fluid;[6] (4 determination of survival time in treated animals;[3, 5, 7, 8, 9] (5) measuremen of the amount of ascites formed after treatment (by visual estimation of th abdominal distention, by weighing mice, or by withdrawing ascitic fluid with syringe and measuring its volume);[1, 7] and (6) more recently, a dye-dilutio

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* This investigation was supported by a grant from the American Cancer Society, New York, N. Y.

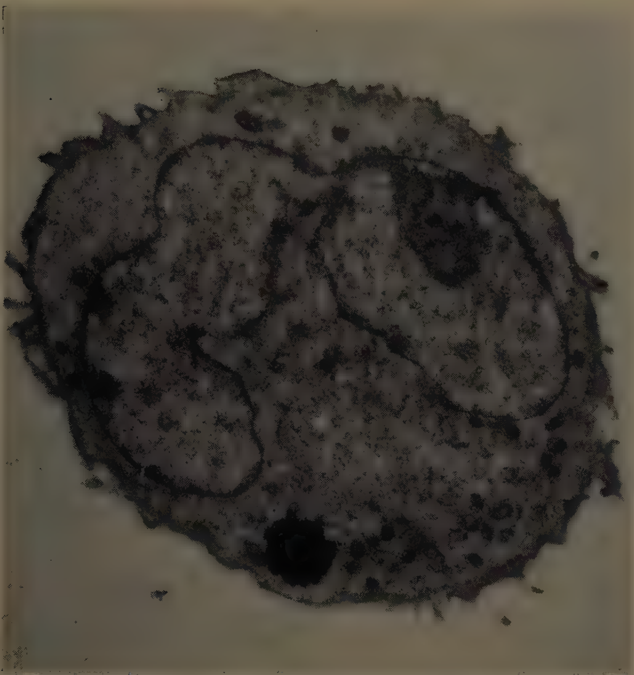

Figure 1. Electron micrograph of Sarcoma 180 ascites tumor showing the cytoplasm, nucleus, chromatin, itochondria, fat, and processes or so-called spikes. Osmic acid fixation. ×6900.

echnique has been introduced to estimate ascitic-fluid volume.[10, 11, 12] We dopted the fifth method.

Chemotherapy test. We adopted the following procedure for chemothera-eutic studies with the ascites tumors. A donor mouse was selected among nice having rapidly accumulating ascites (moderate to marked abdominal listention) at 7 to 14 days after inoculation of cancer cells. About 2 to 5 cc. f milky ascitic fluid were withdrawn with a syringe fitted with a 20-gauge eedle. Then the cells were counted in a hemocytometer, and a proper dilu-ion was made with physiological salt solution at pH 7.0. Intraperitoneal in-ection of 0.1 cc. of the fluid containing about one million cancer cells into young ealthy mice (Rockland Swiss albino mice) weighing 18 to 22 gm. was made in he inguinal region with a 0.25-cc. syringe having a 23-gauge needle. Aseptic recautions were maintained during the procedure. The animals were main-ained on a standard pellet diet (Purina Laboratory Chow) and water.

In each set of experiments, ascites tumor-bearing animals were divided into wo groups, one to be treated with the compounds, and the other to be used as ontrols. The progress of the ascites tumors in the animals was recorded by veight measurements of animals at weekly intervals. In certain experiments, he influence of compounds was evaluated by measuring the amounts of ascitic luid 10 days after the first injection of compounds.

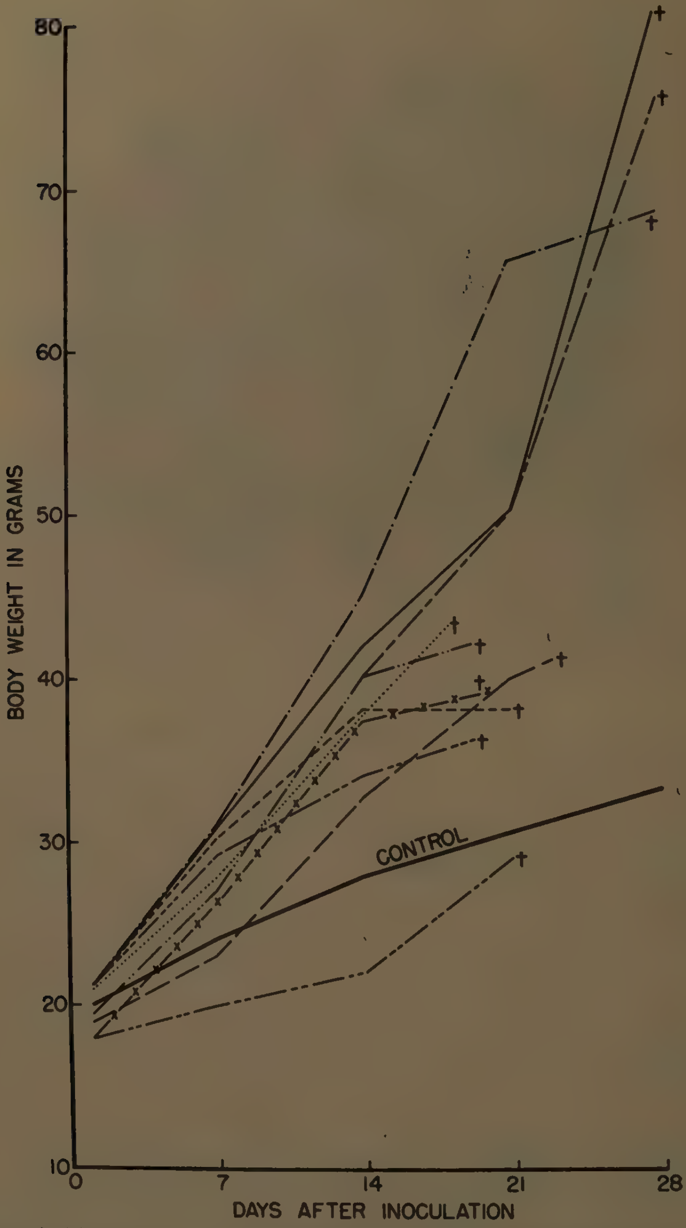

FIGURE 2. Weight curves of mice after injection of one million Ehrlich ascites tumor cells. The heav continuous line (control) indicates the average growth curve of 30 normal young male mice. Each of the oth lines indicates the growth of one ascites-injected young male mouse. Death is indicated by a cross (+).

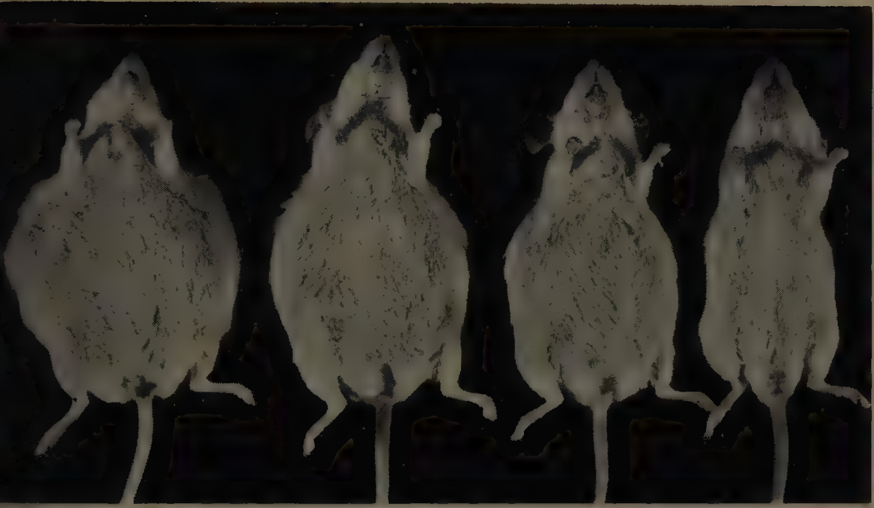

FIGURE 3. The degree of inhibition of ascites tumor growth: — indicates no effect; ± indicates slight inhibition; + indicates moderate inhibition; ++ indicates marked inhibition. (Read from left to right.)

The degree of inhibition of ascites tumor growth was graded accordingly to the following scheme (FIGURE 3):

– No effect, indicating marked abdominal distention; the fluid volume increases to three fourths or more of the volume of the controls (15 to 30 cc.).

± Slight inhibition, indicating moderate abdominal distention; the fluid volume increases to about one half the volume of the controls (5 to 15 cc.).

+ Moderate inhibition, indicating slight abdominal distention; the fluid volume increases to about one fourth the volume of the controls (1 to 5 cc.).

++ Marked inhibition, indicating no abdominal distention; no gross ascites.

+++ Indicates complete inhibition or destruction of ascites.

In some animals, the daily weight increase may be much less due to toxic action of compounds. Survival of more than 50 per cent of the treated animals beyond three weeks was considered a marked effect.

The first intraperitoneal or subcutaneous injection of the maximum tolerated dose of each compound tested was given one day after intraperitoneal injection of about one million cancer cells, and the treatment was continued for seven days.

The injection volumes of the solvents used for the chemotherapeutic agents were as follows: saline (S), 0.5 cc.; carboxymethyl cellulose (CMC), 0.5 cc.; peanut oil (PO), 0.1 cc.; sesame oil (SO), 0.1 cc., all once daily; 0.1 cc. for all subcutaneous treatments.

Results

During recent years, over 100 compounds have been tested against Ehrlich ascites carcinoma (EC), Krebs 2 ascites carcinoma (K2), and sarcoma 180

TABLE 1

EFFECT OF COMPOUNDS ON VARIOUS MOUSE ASCITES TUMORS

Compound	Solvent	Dose mg./kg./day	Effect on ascites tumors		
			Ehrlich carcinoma	Krebs 2 carcinoma	Sarcoma 180
Folic acid	S	50	—	—	—
Aminopterin	S	0.25	±	++	++
A-Methopterin	S	1.0	±	+++	++
Colchicine	S	0.5	—	—	—
Urethane	S	500	+	++	±
Carbamic acid, methyl ester	S	500	—	±	—
N-Methylformamide	S	250	±	+	—
N-Methylacetamide	S	1,500	—	—	—
HN2	S	0.5	+++	+++	+++
HN2 oxide	S	2.0	+++	+++	+++
3-bis(2-Chloroethyl)aminomethyl-4-methoxymethyl-5-hydroxy-6-methylpyridine	S	5.0	+++	+++	+++
Triethylene melamine	S	0.5	+++	+	++
2,4,6-tris(2-Vinylaziridino)-s-triazine	S	2.0	—	—	±
Diethylene phosphoramide	S	4.0	±	—	—
Triethylene phosphoramide	S	6.0	++	++	±
Triethylene thiophosphoramide	S	4.0	++	+	+
1,2-di(Methanesulfonoxy)ethane	CMC	250	+	++	±
1,4-di(Methanesulfonoxy)butane	PO	20	—	++	++
1,9-di(Methanesulfonoxy)nonane	PO	50	+	++	±
Purine	S	250	—	±	—
6-Mercaptopurine	CMC	30	++	++	+++
6-Chloropurine	S	150	±	±	±
Thioguanine	CMC	3.0	+++	±	+
2,6-Diaminopurine lactate	S	60	—	—	—
8-Azaguanine	CMC	75	±	—	±
2,4-Diamino-5-(3′,4′-dichlorophenyl)6-ethylpyrimidine	CMC	10	±	—	±
5-Hydroxyuridine	S	400	++	++	±
Purinyl-6-disulfide	CMC	20	±	++	+
Nebularine	S	5.0	—	—	+
5,7-Dihydroxy-1-v-triazolo(d)pyrimidine	CMC	300	—	—	—
Cortisone	CMC	37.5	++	++	+
Compound F	CMC	37.5	++	++	++
Testosterone propionate	SO	500	—	—	—
Pregnenolone	S	100	—	—	—
Progesterone	S	500	—	—	—
Diethylstilbestrol	CMC	10	—	+	±
S-Diaminobiuret	S	75	—	—	±
Ethionine	CMC	250	—	—	—
2,4-Dimethyl-3-hydroxy-5-hydroxymethyl pyridine·HCl	CMC	125	—	—	—
D-Glucosamine	S	1,500	—	—	—
Podophyllotoxin	Alc. & CMC	2.0	—	—	—
Penicillin	S	2,000	—	—	—
Streptomycin	S	500	—	—	—
Aureomycin	S	50	±	—	—
Chloromycetin	CMC	500	—	—	+
Terramycin	S	150	—	±	±
Azaserine	S	5.0	+	+	+
Actinomycin D	Alc. & S	0.025–0.05	±	+++	+++
Sarkomycin	S	150–250	+++	+++	+++
Fumigillin	CMC	25	+++	++	+
Carzinophilin	S	2,500*	++	+++	+++
Desoxyribonuclease	S	500	+++	—	±
Boric acid	None (in diet)	2,000	++	++	++
Potassium arsenite	S	10	—	—	—

* Units/kg./day

scites tumor (S180). Compounds investigated have been nitrogen mustards, thylene-imins, phosphoramides, folic acid analogs, and other pteridines, car-amates, purines, pyrimidines, formamides, steroids, hormones, enzymes, anti-iotics, antivitamins, inorganic salts, and other compounds of chemotherapeutic nterest.

Results obtained with 54 of the selected compounds on three different mouse scites tumors are presented in TABLE 1. This study included the use of 4,085 ice in the treated group and 1,730 mice in the control group. Each compound as tested, using 5 to 10 mice bearing the ascites tumors, and the experiments ere repeated.

It is apparent from the results that, in 64 per cent of the cases, the responses o agents were identical or almost identical in all three ascites tumors. In the emaining 36 per cent, the responses were of different magnitudes. Thus, folic cid had no effect on the three ascites tumors, but A-methopterin caused a omplete inhibition on K2, a marked inhibitory effect on S180, and a slight hibitory effect on EC. Colchicine was negative on all three ascites tumors, ut urethane showed a marked inhibitory effect on K2, a moderate inhibitory ffect on EC, and a slight inhibitory effect on S180. Nitrogen mustard, HN_2, s oxide, and a methoxy pyridoxin mustard had a complete inhibitory effect on ll three ascites tumors.

HN_2 has a definite damaging effect on the viability of Ehrlich ascites tumor as ell as Krebs 2 and sarcoma 180 ascites tumors at the level of 0.5 mg./kg./day. Vhen mice bearing seven-day-old ascites tumors (showing slight to moderate bdominal distention) were given intraperitoneal injections of daily doses of .5 mg./kg. of HN_2, tumor growth stopped in about two days. Then about 0 per cent of ascites tumors regressed completely in one to two weeks. Ascitic uids tapped from treated mice seven days after the first intraperitoneal in-ection of HN_2 and then injected into normal mice failed to produce ascites. istological examination of abdominal fluids at the end of seven-day treat-ents revealed very few cancer cells, and they were all degenerated (FIGURE 4). or comparison, a smear of untreated Ehrlich ascites tumor cells is shown in IGURE 5.

Triethylene melamine caused a complete inhibition on EC, a marked in-ibitory effect on S180, and a moderate inhibitory effect on K2, but 2,4,6-is (2-vinylaziridino)-S-triazine was completely ineffective on all three ascites umors. Thus addition of three vinyl groups to TEM abolished the carcino-tatic action. Triethylene phosphoramide and triethylene thiophosphoramide ad a moderate to marked inhibitory effect. 1,4-di (Methanesulfonoxy) bu-ane had no effect on EC at 20 mg./kg./day dose level, but inhibited markedly e growth of two other ascites tumors. Two analogs of this compound Myleran) showed different activity. 6-Mercaptopurine exerted a complete hibitory effect on S180 (FIGURE 6), and a marked inhibitory effect on EC and 2, whereas 2,6-diaminopurine had no effect on all three ascites tumors.

Cortisone and Compound F, given subcutaneously, caused a marked in-ibitory effect on all three ascites tumors, but testosterone, given subcutane-usly, had no inhibitory effect. Repeated intraperitoneal injections of 37.5

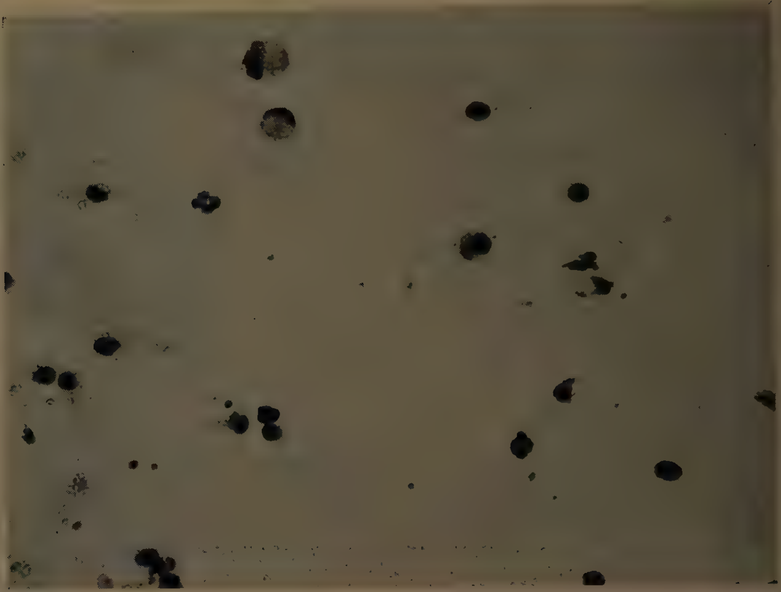

FIGURE 4. Ehrlich ascites tumor after treatment with HN_2 (0.5 mg./kg./day for seven days). The sm shows very few cancer cells, and they are all degenerated. The small cells are normal cells. Papanicola stain. ×400.

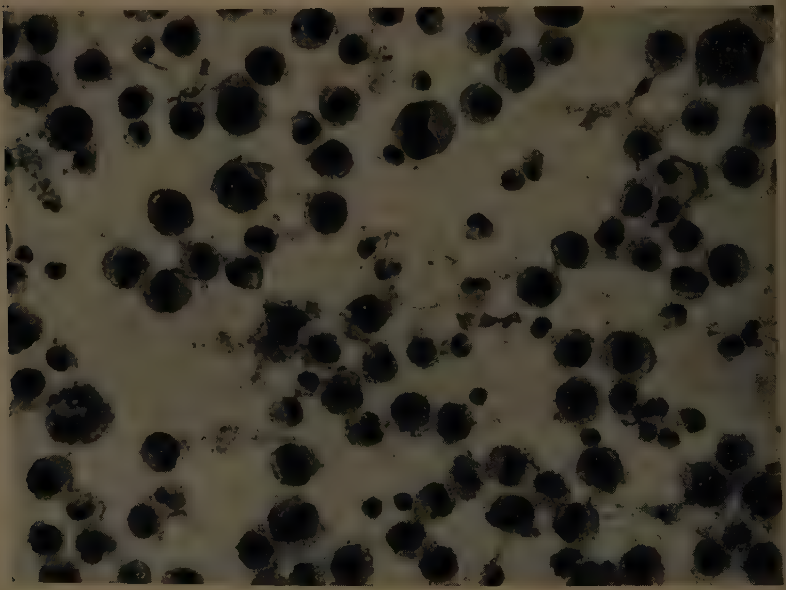

FIGURE 5. A general view of the smear of Ehrlich ascites tumor, showing an abundance of active can cells (large cells) and a small number of normal cells (small cells). Papanicolaou stain. ×400.

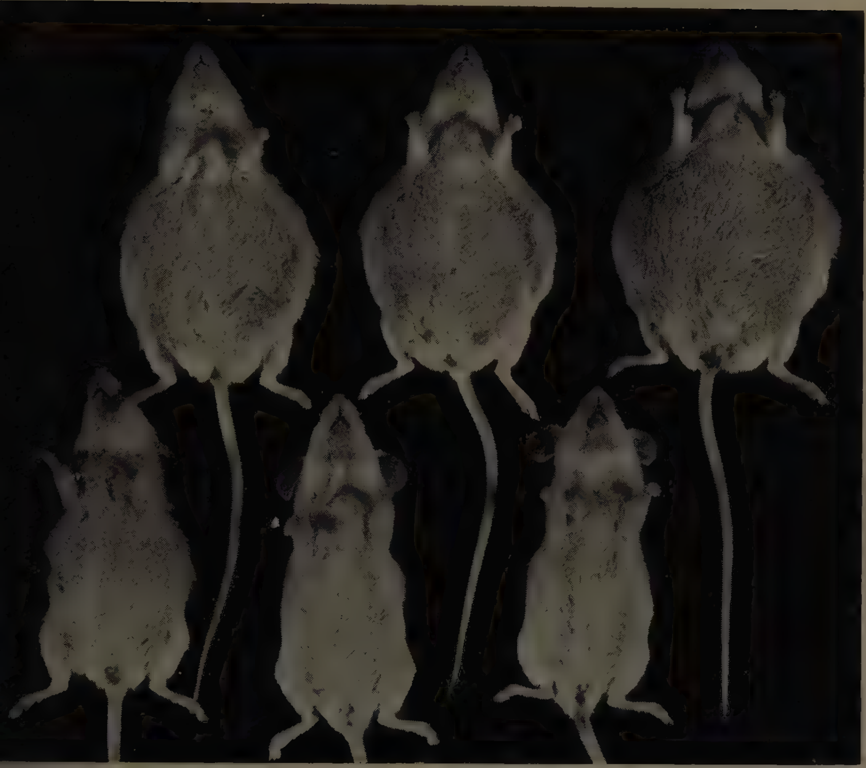

FIGURE 6. The top row illustrates controls with marked distention of abdomen due to rapid growth of Sar-oma 180 ascites tumors. The bottom row illustrates 6-MP treated mice (daily doses of 30 mg./kg. of 6-MP on ven consecutive days). There is no distention of the abdomen, indicating absence of ascites. The animals ere photographed 19 days after inoculation of cancer cells.

g./kg. of cortisone or Compound F had only a slight inhibitory effect on scites tumors.

Penicillin and streptomycin were completely ineffective on all three tumors, ut azaserine had a moderate inhibitory effect. Sarkomycin had a complete hibitory and destructive action on three ascites tumors.[13] Actinomycin D, umigillin and Carzinophilin showed almost similar effect.

Boric acid fed in diet had a definite inhibitory effect on the development of ll three ascites tumors. On the other hand, potassium arsenite given intra-eritoneally had no inhibitory effect.

Subcutaneous injection of all compounds except cortisone and other cortical eroids was less effective than intraperitoneal injection.

As in the case of solid tumors, injection of active compounds five to seven ays after inoculation of the ascites tumors was much less effective than ad-inistration starting one day after inoculation.

A comparison of carcinostatic effects of 54 selected compounds on solid and scites forms of Ehrlich carcinoma and sarcoma 180 is presented in TABLE 2.

Table 2

Effect of Various Compounds on Solid and Ascites Forms of the Same Tumor

Compound	Dose mg./kg./day	Ehrlich carcinoma		Sarcoma 180	
		Solid	Ascitic	Solid	Ascitic
Folic acid	50	—	—	—	—
Aminopterin	0.25	±	±	++	++
A-Methopterin	1.0	—	±	+	++
Colchicine	0.5	—	—	—	—
Urethane	500	—	+	+	±
Carbamic acid, methyl ester	500	—	—	—	—
N-Methylformamide	250	+	±	±	—
N-Methylacetamide	1,500	±	—	—	—
HN2	0.5	±	+++	±	+++
HN2 oxide	2.0	—	+++	±	+++
3-bis(2-Chloroethyl)aminomethyl-4-methoxymethyl-5-hydroxy-6-methylpyridine	5.0	±	+++	±	+++
Triethylene melamine	0.5	±	+++	+	++
2,4-6-tris(2-Vinylaziridino)-s-triazine	2.0	—	—	—	±
Diethylene phosphoramide	4.0	—	±	±	—
Triethylene phosphoramide	6.0	+	++	+	±
Triethylene thiophosphoramide	4.0	+	++	+	+
1,2-di(Methanesulfonoxy)ethane	250	+	+	±	±
1,4-di(Methanesulfonoxy)butane	20	±	—	—	++
1,9-di(Methanesulfonoxy)nonane	50	±	+	—	±
Purine	250	±	—	±	—
6-Mercaptopurine	30	+	++	+++	+++
6-Chloropurine	150	±	±	+	±
Thioguanine	3.0	+	+++	+	+
2,6-Diaminopurine	60	±	—	—	—
8-Azaguanine	75	—	±	—	±
2,4-Diamino-5-(3′,4′-dichlorophenyl)6-ethylpyrimidine	10	±	±	±	±
5-Hydroxyuridine	400	±	++	±	±
Purinyl-6-disulfide	20	+	±	+	+
Nebularine	5.0	—	—	±	+
5,7-Dihydroxy-1-v-triazolo(d) pyrimidine	300	—	—	—	—
Cortisone	37.5	±	++	—	+
Compound F	37.5	+	++	±	++
Testosterone	500	—	—	—	—
Pregnenolone	100	—	—	—	—
Progesterone	500	—	—	—	—
Diethylstilbestrol	10	—	—	—	±
S-Diaminobiuret	75	—	—	—	—
Ethionine	250	—	—	—	—
2,4-Dimethyl-3-hydroxy-5-hydroxymethyl pyridine·HCl	125	+	—	—	—
D-Glucosamine	1,500	—	—	—	—
Podophyllotoxin	2.0	—	—	—	—
Penicillin	2,000	—	—	—	—
Streptomycin	500	—	—	—	—
Aureomycin	50	—	±	—	—
Chloromycetin	500	—	—	—	±
Terramycin	150	—	—	+	±
Azaserine	5.0	±	+	++	+
Actinomycin D	0.025–0.05	±	±	—	+++
Sarkomycin	150–250	±	+++	—	+++
Fumigillin	25	++	+++	+	+
Carzinophillin	2,500*	—	++	—	+++
Desoxyribonuclease	500	—	+++	—	±
Boric acid	2,000†	+	++	+	++
Potassium arsenite	10	—	—	—	—

* Units/kg./day
† Fed

When the responses of the solid tumors and ascites tumors to the 54 compounds are matched, it is found that they agree in only 23 of 54 instances (43 per cent) in the case of Ehrlich carcinoma, and 27 of 54 instances (50 per cent) in the case of sarcoma 180. In practically all cases, the ascites tumors were more sensitive to the carcinostatic action of agents than their respective solid tumors. In a few cases, solid tumors responded better than their respective ascites tumors as Ehrlich carcinoma treated with N-methylformamide, N-methylacetamide, purinyl-6-disulfide, and 2,4-dimethyl-3-hydroxy-5-hydroxymethyl pyridine·HCl, and sarcoma 180 treated with urethane, N-methylformamide, diethylene phosphoramide, triethylene phosphoramide, purine, 6-chloropurine, terramycin, and azaserine.

The greater sensitivity of ascites tumors to the same carcinostatic agents, as compared with the sensitivity of their corresponding solid forms, is not due to the use of relatively newly developed tumors.* This is seen clearly by the following comparative experiments involving 160 mice, exclusive of controls. One group of 10 animals was implanted subcutaneously with solid tumor fragments, and another group of 10 animals was injected subcutaneously with ascitic fluid containing about one million cancer cells. The following day, these animals received intraperitoneal injections of 0.5 mg./kg. of HN_2 or 0.5 mg./kg. of triethylene melamine, and treatment was continued for seven days. The results of this experiment showed that the effectiveness of HN_2 and TEM against solid tumors developed from tumor fragments or ascites tumors was the same—HN_2 had no effect on Ehrlich carcinoma from either source and a slight inhibitory effect on sarcoma 180 from either source, while TEM had a slight inhibitory effect on Ehrlich carcinoma from either source, and a moderate inhibitory effect on sarcoma 180 from either source.

A further comparison of the sensitivity of ascites tumors and solid tumors to the same carcinostatic agents is given in the recent publication of the "Investigation of Diverse Systems for Cancer Chemotherapy Screening" by Gellhorn and Hirschberg."[14] In table 3 (a summary of all results) of this paper,[14] Compound 6 (6-mercaptopurine) produced a slight inhibitory effect on the EF Ehrlich ascitic tumor. Compound 24 (colchicine) caused a marked inhibition of this tumor, and Compound 28 (azaserine) showed no effect. With our Ehrlich ascites tumor, the ES of Doctor T. S. Hauschka (which kills mice more slowly than the EF), we have observed marked inhibition with 6-mercaptopurine, no effect with colchicine, and moderate inhibition with azaserine. These discrepancies (between Doctor H. J. Creech's findings and ours) are, presumably, due to the use of two different lines of the Ehrlich ascites tumors, or due to different preparations of colchicine. Crystalline colchicine kindly supplied by Professor Hans Lettré had a destructive effect on Ehrlich ascites carcinoma and sarcoma 180 ascites tumor, but had practically no inhibitory effect on the respective solid tumors. Our cochicine was amorphous.

It is interesting to note that repeated intraperitoneal injections of Sarkomycin had a destructive effect on all three ascites tumors. This antibioti however, had practically no inhibitory effect on the respective solid tumor Intensive clinical trials of this drug have also indicated no carcinostatic effec on various types of cancers in the human subject.[15]

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* Ehrlich mouse carcinoma has been continuously propagated by subcutaneous transplantations since 1906, and Crocker mouse sarcoma 180 since 1914. Ehrlich ascites carcinoma has been continuously propagated by intraperitoneal injections since 1950, and sarcoma 180 ascites tumor since 1952.

Discussion and Summary

Quantitative evaluation of results in chemotherapy studies with ascite tumors has not yet been placed upon as definite a basis as similar investigation with solid tumors. The formation of solid tumors at the site of injection alon with the ascites may complicate the evaluation, whether it is based upon weigh of the mouse or upon increase in survival time. It has been suggested[5] tha treatment with chemicals may decrease the amount of ascitic fluid withou significantly influencing the number of tumor cells. In such cases, misleadin information would be obtained from weight measurements. This possib source of error, however, can be detected by total cell counts.

Comparison of carcinostatic effects of 54 compounds on both ascites and soli forms of the same mouse tumors has demonstrated that the ascites forms appea to be the more sensitive system for detection of antitumor activity. Intra peritoneal injection of chemicals into ascites tumor-bearing mice, however, rep resents a form of intratumoral treatment and, as such, it has possibly le significance than treatment of solid tumor growing at a distance from the inje tion site.

Acknowledgments

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Part II

By Hugh J. Creech

Almost two years ago, Doctor Hauschka and I were asked to participate in a liverse systems screening program initiated by the American Cancer Society, Vew York, N. Y. Our project involved testing 27 selected compounds on scites tumors. Some of the compounds were known to possess antitumor ctivity, some were otherwise biologically active, and others were inactive nalogs. As the time allowed for this study was limited, we selected survival ime as a rigorous and practical criterion and supported it, in certain instances, y cell counts.

Since Doctor Hauschka had previously collected much information on var-ous ascites tumors, we decided to compare the effects of the 27 compounds on our cytologically distinct lines of the Ehrlich ascites tumor that exhibited ifferent degrees of virulence. After inoculation with two to three million ells, male Swiss mice bearing two hypotetraploid lines with chromosome nodes of 75 showed mean survival times of 13.0 ± 0.30 and 11.1 ± 0.27 days. Jnder the same conditions, mice bearing a hyperdiploid line (46 chromosome node) died in 17.6 ± 0.26 days, and those inoculated with a hypertetraploid ne (92 chromosome mode) died in 16.7 ± 0.29 days.

After determination of the maximum tolerated dosage, each compound, dis-olved in 0.2 to 0.5 ml. of physiologic saline, or in 1 per cent carboxymethylcellu-ose solution when necessary, was injected intraperitoneally one, two, and hree days after tumor inoculation. Control mice were injected with equiva-ent amounts of the appropriate solvent. Several of the compounds were also njected at later intervals. The survival data were graphed daily and, to ermit a conservative evaluation, the experiments were terminated upon the eath of 90 per cent of the mice in the control and experimental groups. Based n the more than 4,000 mice used in our tests, an analysis of the variability f mean survival time between groups showed that a difference of 20 per ent could be considered statistically significant.

Since the detailed results of this study have been published recently (Creech, Iauschka, Hankwitz, Littleton, and Andre,[1] we shall outline only the major observations and pass on to a comparison of our results with those of othe investigators using ascites and solid tumors. Under our specific experimenta conditions, nitrogen mustard and colchicine prolonged the survival time o mice bearing each of the four ascites tumors by 75 to 350 per cent. Increase in survival time of 25 to 40 per cent with some, but never all, of the four line of Ehrlich ascites tumor, were caused by Amethopterin, N-methylformamide urethane, potassium arsenite, 6-mercaptopurine, 8-azaguanine and 2,6-diami nopurine. The rapidly killing hypotetraploid Ehrlich tumor clone (Hauschka was more susceptible than the slower killing hypotetraploid ascites stoc (Klein) from which it had been derived. The very slowly killing hyperdiploi Ehrlich ascites tumor (Lettré) and its hypertetraploid derivative (Kaziwara did not differ distinctly from each other. They were, however, the least sensi tive of the four tumor lines. Injections of active compounds beginning one da after tumor inoculation were much more effective than later injections. A increase in survival time was generally accompanied by a proportional decreas in ascites cell count per mouse. The solvent carboxymethylcellulose cause an appreciable increase in survival time with three of the tumor lines.

In TABLE 1, there is presented a comparison of the responses of ascites an solid tumors with the over-all ratings obtained with 15 tumor systems (Gell horn and Hirschberg.[2] With the exception of colchicine, the 11 importan chemotherapeutic agents listed in this table were classified as positive in th screening project. Our results with ascites tumors are listed in the secon column, with a ++ value being assigned to compounds that increased the mea survival time of the mice by greater than 125 per cent, a + value to increase of 51 to 125 and a ± value to increases of 20 to 50 per cent. A — value wa given to increases of less than 20 per cent. The next two columns show whic investigators agreed or disagreed with the over-all ratings on the basis of thei studies involving survival time, or cell counts, or ascites growth measurement

TABLE 1

COMPARISON OF ASCITES AND SOLID TUMORS WITH THE OVER-ALL SCREENING AVERAGE

Compound	Over-all screening average	Ascites tumors			Solid tumors	
		Creech *et al.* average	Agreement with over-all rating		Per cent positive	Agree ment with over-a averag
			yes	no		
Nitrogen mustard	Pos.	++	C.Y.K.S.		67	yes
Colchicine	Neg.	+	S.	C.Y.K.L.	17	yes
Amethopterin	Pos.	±	C.K.S.L.	Y.	25	no
N-Methylformamide	Pos.	±	C.S.		67	yes
Potassium arsenite	Pos.	±	C.Y.	S.	25	no
2,6 Diaminopurine	Pos.	±	C.	S.	25	no
6-Mercaptopurine	Pos.	—	S.	C.Y.	92	yes
Urethane	Pos.	—	S.	C.Y.	67	yes
8-Azaguanine	Pos.	—	S.	C.Y.L.	67	yes
Myleran	Pos.	—		C.S.	50	yes
Azaserine	Pos.	—	S.LeP.	C.	58	yes

C. = Creech *et al.*, K. = Klein[3], L. = Lettré[4, 5], LeP. = LePage[6], S. = Sugiura[7, 8], Y. = Yoshida[9, 10].

or cytological examination for nuclear damage. We found that the first six compounds caused significant prolongation of survival time of mice bearing two or more of the ascites tumor lines. The majority of the other workers agreed with our findings. With 6-mercaptopurine, urethane, and 8-azaguanine, our results showed an average negative response, although one tumor line was positively influenced by each compound. Azaserine and Myleran (1,4-dimethanesulfonyloxybutane) were negative under our experimental conditions. Sugiura[8] and LePage[6] considered azaserine to be positive; the latter investigator found a ± prolongation of survival time when the compound was injected twice a day for six days at a level of 1 mg./kg./day/mouse.

It must be emphasized that our results were obtained under one strict set of experimental conditions established for the conservative evaluation of the responses of mice bearing four distinct lines of the Ehrlich ascites tumor. We injected maximum tolerated doses of the compounds one, two, and three days after tumor inoculation. Although ascites tumors, following inoculation of two to three million cells, are considered to be satisfactorily established by one day, an even more important comparison might have been obtained by injecting the compounds either three, four, and five, or two, three, and four days after inoculation of the ascites tumor. It seems reasonable to conclude that all our statistically significant positive results are valid but that, under other conditions of experimentation involving different mice, tumors, numbers of ascites cells inoculated, dosages of compounds, schedules of administration, and criteria for judging the response, some of the compounds, which we found to be weak or inactive, may exert a definite restricting influence on the course of development of ascites tumors.

The next comparison involves the over-all screening results and those obtained with the 12 solid tumors. Details on the solid tumors and analytical methods are contained in the previously mentioned report[2] edited by Doctors Alfred Gellhorn and Erich Hirschberg. Positive compounds were considered to be those that affected 33 per cent or more of the 15 tumor systems under study in the program. All the clinically useful compounds fell in this category. In the table of the present paper, the values for the solid tumors were calculated from the data in the screening report.[2] The heading "per cent positive" gives the percentages of the 12 tumors that were affected by the various compounds according to the criteria established by the different investigators. Again, taking the 33 per cent level as the "cut-off point,"[2] it is seen that four of the 11 listed compounds were negative against solid tumors under the experimental conditions employed. Only two of the 12 solid tumors were well established in growth at the time the compounds were injected. Intraperitoneal injection of the compounds was begun 24 hours after tumor transplantation in the studies with 10 of the tumor types. A conspicuous lack of agreement is immediately apparent between our critical evaluation with the ascites tumors and the results with the solid tumors, which were composite ratings involving tumor regression and interference with tumor takes. Complete agreement was reached only with nitrogen mustard and N-methylformamide. Of the 10 compounds judged to be positive in the over-all analysis, our results with ascites tumors showed five positive; Sugiura had seven positiv results in his work with ascites tumors, and the solid tumors showed seve positive.

Considering all 27 compounds, however, 12 were considered negative in th over-all screening. Our one disagreement with this list was colchicine. Of th remaining 15 positive compounds, our results showed 6 positive with mor than one ascites tumor line and 9 positive with one line. Comparison of each of the 15 tumor systems with the average response (15 positive and 12 negativ compounds) showed no perfect matching score for any one tumor. The bes tumor was 89 per cent matching, the worst was 59, the average was 70 per cent. and our two ascites tumor lines (Hauschka hypotetraploid clone and Lettr hyperdiploid), which were included in the over-all analysis, had 67 and 63 pe cent matching scores.[2]

A few rather evident conclusions can be drawn from these studies. First as has been shown by others, this work confirms the idea that no one tumo can be expected to constitute an adequate screen for potentially active compounds. Also with any one tumor, no one set of experimental conditions, no any one criterion, can be expected to find every compound with some activit against that tumor. A single standardized screening system may often b either too coarse or too fine. Too critical a screen, using thoroughly established tumors and extremely strict criteria, may be just as faulty as a sensitiv screen that detects compounds that interfere with tumor takes (hence, c probable importance to metastases) but do not necessarily cause regression Judicious use of both types of screen is obviously desirable—the sensitive on for preliminary work and the critical one for demonstration of truly significan activity of a compound. The ascites tumors provide an excellent system fo quantitative measurements, provided statistical analysis is applied to surviva time and cell counts to avoid assignment of + or ± ratings to changes tha are within the limits of biological variation. Although the ascites tumors, a we employed them, do not appear to offer general advantages over solid tumor as a single screening system, the detection of activity in certain classes of compounds, such as derivatives of nitrogen mustard and colchicine, may be aide by the incorporation of ascites tumors into a multiple systems screen. Comprehensive studies involving detailed cytological examinations and a searc for modes of action of chemotherapeutic agents might well take advantage c the merits of ascites tumors.

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Part IV. Advantages of Free-Cell Suspensions for Metabolic Studies.

COMPARATIVE METABOLIC STUDY OF MC1M ASCITES AND A SUSPENSION OF WHOLE CELLS OBTAINED FROM MC1M SOLID TUMOR

By John Paul Kaltenbach* and Merle H. Kaltenbach
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This monograph deals with the ascites cell as a tool in cancer research. This particular presentation, however, is to describe a relatively new tool that may be termed an analogue of ascites-cell suspensions, namely, suspensions of whole cells from solid tissue. The use of ascites cells in biological studies offers advantages previously limited to bacteria and other cells already in a free state. It has been repeatedly demonstrated that ascites tumor cells are a valuable tool for quantitative investigation of such fundamental processes as growth rate, mitotic activity, morphology, and metabolism. It would appear, therefore, that a suspension of whole cells from normal tissue such as liver, or neoplastic tissue such as solid tumors would be of similar value in obtaining such basic information.

The purpose of this paper is to describe briefly the method for obtaining suspensions of whole cells from tissues such as liver and solid tumors, and to present several aspects of the metabolism (glycolysis) and survival of cells obtained from the solid form (M/AS) and the ascites form (M/AA) of the MC1M tumor.†

The preparation of liver-cell suspensions has recently been reported by one of us,[1] and this same procedure is applicable to tumor cells. Somewhat diferent sizes of screens may be used, depending upon the fibrous or nonfibrous nature of the tumor, and the size of the tumor. The procedure, in brief, is as follows. The tissue is excised and placed in a cold isotonic medium. Each obe of the liver, or each small tumor, is halved, and the cut surface placed against a 14- to 20-mesh (depending upon the tissue) stainless steel screen. The tissue is gently forced through the screen with a washed, soft rubber stopper. The tissue is resuspended, and forced through a second screen (24 to 60 mesh), again resuspended, and finally poured through an 80 to 100 mesh screen. The use of several sizes of screens serves to break up the solid tissue for each succeeding screen, so that the pressure exerted upon the cells passing through the screen is minimized. The screens also serve to retain much of the blood vessels, capsule, and cellular debris

The criteria applied in determining the integrity of the cells in suspension were of two types: morphological and biochemical. The morphological crieria included microscopic examination of fixed and unfixed smears, and the

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* This investigation was supported by a research fellowship from the National Cancer Institute of the Naional Institutes of Health, United States Public Health Service, Bethesda, Md., and was carried out at the nstitute for Cell Research and Genetics, Karolinska Institute, Stockholm, Sweden.

† M/SS designates the original solid line of the MC1M tumor kept by serial subcutaneous transplants. M/AA esignates the ascites form obtained from M/SS by serial intraperitoneal transplantation of peritoneal exudate. M/AS designates the solid tumors produced by subcutaneous inoculation of the ascitic fluid.[2, 3]

TABLE 1

THE LACK OF GLYCOLYTIC ACTIVITY OF STAINED CELLS

	Modified* ascites cells ($\times 10^6$)		Ascites cells ($\times 10^6$)
	5.3 Unstained cells 4.7 Stained cells 10.0 Total cells	10.0 Unstained cells 8.9 Stained cells 18.9 Total cells	10.0 Unstained cells 0.2 Stained cells 10.2 Total cells
μM lactic acid	2.8 2.2	4.8 5.0	5.6 5.0

* Modified cells: ascites cells kept in a hypotonic medium until approximately 50 per cent of the cells were stained, then medium again made isotonic.

These data are representative of four separate experiments done in duplicate.

determination of living and dead cells* in suspension by the method of unstained cell counts.[7] Little of the metabolic data obtained from ascites cells has been expressed on a per-cell or per-live-cell basis. This may not be too important in the case of ascites cells that normally have 90 per cent, or more, living cells. Suspensions of cells obtained from solid tissue, however, may have a range of 15 to 70 per cent live cells. Since glycolytic activity was found to be dependent upon the number of live cells, rather than total cells, the determination of the number of live cells is important in these studies.

Evidence indicating the lack of activity of dead (unstained) cells is shown in TABLE 1. Cell suspensions containing 10 × 10^6 live cells and suspensions containing 10 × 10^6 live cells plus 8.9 × 10^6 dead cells had similar activity. Thus, the glycolytic activity was due only to the live cells present. Modification of the cells as described in TABLE 1 had no apparent effect on their activity. The validity of the unstained cell-count method had never previously been demonstrated applying metabolic criteria. The results illustrated in TABLE 1 indicate that the method is valid under the conditions in which it was employed, *i.e.*, the glycolytic activity was proportional to the number of unstained cells. Because of the wide difference in the ratio of live to dead cells that may be obtained from various solid tissue, the activity on a per-live-cell basis is more valid than that according to dry weight or nitrogen.

Of the biochemical criteria that indicated the functional integrity of the cell suspensions,[1] the incorporation of radioactive glycine into cell proteins, anaerobically, was most conclusive. LePage[5] has demonstrated that a suspension of whole cells has a sevenfold greater incorporation of radioactive glycine than did a fortified[4] cell homogenate. These results indicated the integrity in the whole cells of the enzyme systems required for anaerobic incorporation of radioactive glycine. Since the energy requirement[5, 6] did not appear limiting, it would seem that these enzymes were also intact.

While we were working with Doctor Klein at the Karolinska Institute, the technique of preparing suspensions of whole cells from solid tissue was applied to tumor tissue. The tumor used in these experiments was an MC1M methylcholanthrene-induced sarcoma. This tumor was chosen because it may exist in three forms, classified with regard to its convertibility into ascites tumors.[2,

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* For convenience, cells not stained by eosin will be referred to as live cells, and cells stained by eosin will be termed dead cells.

Comparing the AS and the ascites form of this tumor was of primary interest. As the AS may be converted into either the ascites or the solid form, it may offer a means of investigating metabolic adaptation in mammalian cells. The following is a description of the procedure and results of experiments comparing the glycolytic metabolism and survival, *in vitro*, of suspensions of cells from the M/AS and M/AA forms of the MC1M tumor.

In the experiments using tumor cells, standard Warburg technique was used. Anaerobic and aerobic glycolysis was quantitated by determining the lactic acid formed. Each flask contained modified Ringer's medium,[1] 0.5 per cent glucose, and 5 × 10^6 live cells. Total volume per flask was 3.0 ml. The atmosphere in the flasks was either 5 per cent CO_2-air or 5 per cent CO_2-N_2, as indicated. Cell counts were made before and after each experiment. At the end of all experiments, an aliquot from each flask was removed for lactic acid determination, and a cell count was made on the remaining material.

As the solid AS form of tumor has a different environment in the mouse than has the ascites form, experiments were conducted to determine whether or not this environmental difference would result in a difference in metabolism of the two tumors. The results shown in TABLE 2 indicate that the aerobic glycolytic metabolism of the AS and the ascites cells is similar. Of the two, however, the AS cells had only about 60 per cent of the anaerobic glycolytic activity of the ascites cells. The percentage of survival of the AS cells, however, was almost twice that of the ascites cells. Since the results shown in TABLE 1 indicate that only the live cells contribute to the glycolytic activity, it would appear that the ascites cells either have a higher rate of metabolism, or that their metabolic rate was high because of their low survival; *i.e.*, that an increase in metabolism is often characteristic of dying cells.

Discussion

The use of suspensions of whole cells obtained from solid tumor tissue allows comparative morphological and metabolic studies to be carried out under conditions previously limited to ascites cells. The amount of injury attributable to the screening procedure would be difficult to assess. The results shown in TABLE 2, however, indicate comparable activity between the cells obtained from solid tumor and ascites. Moreover, it was previously shown that the succinoxidase activity of screened liver cells was only slightly less than homogenates,[1] and somewhat higher than liver slices. It appears, therefore, that injury to the live (unstained) cells is not significant.

TABLE 2

GLYCOLYTIC ACTIVITY AND SURVIVAL OF MC1M/AS AND MC1M/ASCITES CELLS

Tumor	No. of Expts.	μM Lactic/hr.		Lactic inhibit. by air	% Survival	
		5% CO_2-air	5% CO_2-N_2		5% CO_2-air	5% CO_2-N_2
AS..................	22	4.3	5.8	25.8%	77.6%	81.4%
Ascites.............	10	4.7	8.0	36.9%	47.0%	41.6%

The yield of cells per gram of tissue from the two types of solid tumors wa found to be quite different. The M/SS appeared to be more fibrous than th M/AS form; *i.e.*, more M/SS tissue was retained by the screens than M/AS However, on the basis of equal desoxypentosenucleic acid values per gran solid tumor for M/SS and M/AS, Doctor Eva Klein concluded that the differences in yield of cells does not reflect a variation in the number of cells pe gram solid tumor.[3] The large proportion of spindle-shaped cells in the M/SS may explain partially the differences in the yield of cells from the two tumors *i.e.*, round cells would be easier to screen than spindle-shaped cells. The difference in the number of cells obtained per gram of tumor is indicated in FIGURE 1. Approximately three times as many cells were obtained from th

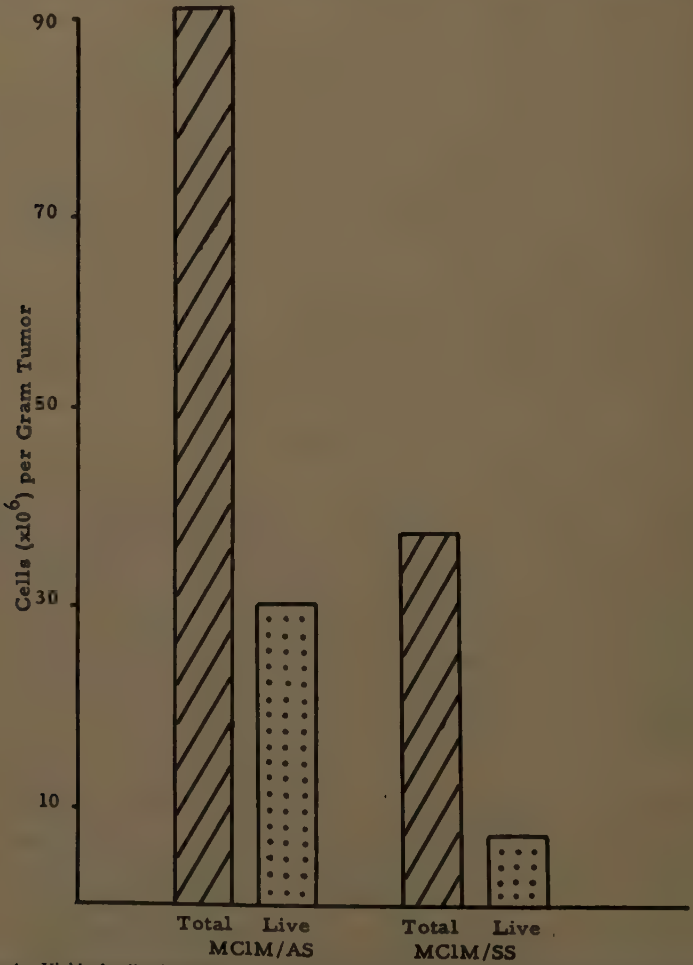

FIGURE 1. Yield of cells obtained by screening solid tumors. Results are averages obtained from 22 A and 15 SS tumors.

S than from the SS tumors. Also, a greater percentage of live cells per ram of tumor was obtained from the AS than from the SS tumor (SS tumors ere used for only a very few metabolic experiments because of the small num-er of live cells per gram of tumor that could be obtained).

The glycolytic activity of the MC1M ascites cells was comparable to the ctivity of Ehrlich ascites cells.[5] However, approximately 90 per cent or better f the Ehrlich cells survive one hour incubation, whereas about 50 per cent of he MC1M ascites cells survive (unpublished data). The variation in sur-ival of different ascites cells has also been observed by Doctor R. Schrek private communication). We also observed that the ascites cells had a greater endency to clump under aerobic conditions than did the AS cells. The lumping of ascites cells, aerobically, was also observed by Doctor Hans ettré (private communication). This clumping phenomenon, usually asso-iated with dead or dying cells, may be a factor causing low aerobic activity ith some ascites cells. Definitive conclusions will have to await additional tudies.

Summary

(1) The technique of preparing suspensions of whole cells from solid tissue as described and its application to solid tumors was discussed.

(2) The aerobic and anaerobic glycolytic activity and survival of M/AS ells was compared with that of M/ascites cells.

(3) The metabolic activity of cell suspensions was demonstrated to be due o the whole live (unstained) cells present.

(4) Metabolic evidence was presented indicating the validity of the un-tained cell-count method for determining cell viability.

Acknowledgments

The authors wish to express their appreciation to Doctor George Klein for is suggestions and cooperation, to Doctor Eva Klein for our supply of tumors, nd to Doctor T. Caspersson for his interest in this investigation. We also ppreciated the kind help given us by all staff members of the Karolinska nstitute.

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Discussion of the Paper

DOCTOR GERALD A. LEPAGE (*University of Wisconsin, Madison, Wis.*): In is paper "Conversion of Solid Neoplasms into Ascites Tumors," Doctor Klein as commented (p. 13-Ms) that the growth of ascites tumor cells appears to ccur under very unfavorable nutritional conditions and that, in contrast, the cells of a growing solid tumor are in contact with a blood supply. At least, during the earlier phases of growth, these cells of solid tumors are relatively well nourished. Indeed, the ascites cell form appears to be a selection of cells able to thrive in such poor nutritional conditions.

This makes it important that we shall be able to make comparisons of cells from solid tumors with those of the ascites cell form, on the basis of biochemical characteristics, in such a manner that they are on an equal footing. The technique that Doctor Kaltenbach has described seems the best solution to the technical problem of making such a comparison. His choice of the MC1M tumor, which was available in the three different forms, all of a common origin, is an additional, desirable feature.

Doctor Kaltenbach has used glycolytic activity as a biochemical criterion for comparing the ascites cells with suspensions prepared from solid tumors. Since these glycolytic reactions are probably the major source of energy for growth of these cells, such a comparison is very pertinent. Here, the ascites cells appear to have some advantage; *i.e.*, to have a somewhat greater glycolytic rate.

It is easy, of course, to suggest other biochemical criteria upon which the cell suspensions might be compared. One that comes of mind immediately is the ability to concentrate nutrients, a feature that would be expected to relate directly to ability to grow in poor nutritional conditions. It would be interesting to compare the cell suspensions on this basis. It seems appropriate to leave consideration of this point to the following paper by Doctor Halvor N. Christensen.

CONCENTRATIVE UPTAKE OF AMINO ACIDS BY FREE-CELL NEOPLASMS*

By Halvor N. Christensen, Thomas R. Riggs, Anita J. Aspen, and Stella Mothon
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This report concerns a cellular activity discovered in 1913 by Van Slyke and Meyer,[1] namely, an ability to transfer amino acids into the cell interior against apparent concentration gradients. Although this activity is possessed by a great variety of cells, it is particularly strong in neoplastic tissues, and the Ehrlich mouse-ascites tumor cells have been selected for the studies to be discussed here. Much of what is known about the process in these and other cells of mammalian origin has been reviewed recently[2] and will be summarized briefly here. Studies of a similar activity of bacterial cells also have been reviewed.[3]

This concentrative uptake is the first event in the cellular utilization of the amino acids. We have considered also whether the rate of protein synthesis and of growth in each tissue may be determined, in a degree, by the intensity of the tissue in concentrating amino acids. In general, one would suppose that wherever growth is accelerated, lowered amino acid levels would be found. Instead, higher amino acid levels repeatedly have been found in such sites. A particularly interesting example is the high amino acid levels of fetal tissues, a consequence of increased concentration across the fetal barrier and of increased concentration by fetal cells.[4]

Another example, very recent, comes from the findings of Miller and Burke.[5] The amino acid levels of the livers of carcinogen-treated rats were found to be ncreased before the hepatoma actually appeared.

Our analyses indicate that the Ehrlich ascites tumor cells are nourished by he amino acids that diffuse passively into the fluid in the peritoneal space.[6] The host tissues are able to give up amino acids so rapidly that the ascitic plasma is only very slightly depleted as far as amino acids are concerned. In ontrast, the tumor cells severely deplete the glucose of the fluid, as Doctor Klein has reported elsewhere in this monograph.

Analysis of the tumor cells gives an even stronger picture of prosperity ather than of starvation, again as far as the amino acids go. The neoplastic ells concentrate amino acids with exceptional avidity. After removal from he host, they continue to do this with undiminished vigor, although net rotein synthesis soon ceases. It may be mentioned that the persistent high ndogenous oxygen consumption by the isolated tumor cells indicates that these ells have been able to accumulate energy-yielding nutrients also during their rowth.

It should be emphasized that, in spite of the presence of an apparently bundant supply of nutrients in the cells, their growth is probably still retrained because the level of these nutrients is not still higher. The growth f the tumor is undoubtedly governed by competition with the other tissues of

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* Assisted by a grant (C-1268) from the National Cancer Society, National Institutes of Health, United States ublic Health Service, Bethesda, Md., and by a grant from the Abbott Laboratories, N. Chicago, Ill.

the mouse for nutrients. The competitive advantage of the tumor cell is probably expressed by its higher accumulation of these nutrients. Another limitation, undoubtedly, is the rate at which the mouse can acquire extracellular electrolytes to form the necessary volume of ascitic plasma.

The Ehrlich ascites tumor cells were selected for study because of their resistance to injury and convenience of handling, as well as for their particularly strong concentrating activity. Strong concentration appears to be characteristic of the rapidly growing tumors that have been studied. It should be emphasized, however, that the purpose has been to study a normal metabolic process by use of a tumor cell, rather than to investigate the special biochemistry of neoplastic cells.

By an energy-requiring process, the Ehrlich ascites-tumor cell is able to develop apparent amino acid gradients as high as 140 millimolar. A proportionate transfer of water speaks for the reality of the gradients. So, also, does the very size of the gradients, because any possible binding agent would have to be present in improbable quantities to hold so much extra amino acid in the cells in a loosely-bound form. Kinetic studies have shown that glycine transferred into the cells becomes bound to an acceptor of limited capacity as a rate-limiting step in its uptake, but that it does not remain bound to this substance within the cells.[7, 2] Furthermore, the efflux of an accumulated amino acid is proportional to the amount accumulated. Finally, the existence of a true active transport for amino acids is established by the known ability of the renal tubular cells, the intestinal mucosa, and the placental barrier[4] to concentrate amino acids.

The transfer process may conceivably occur as illustrated in FIGURE 1. The

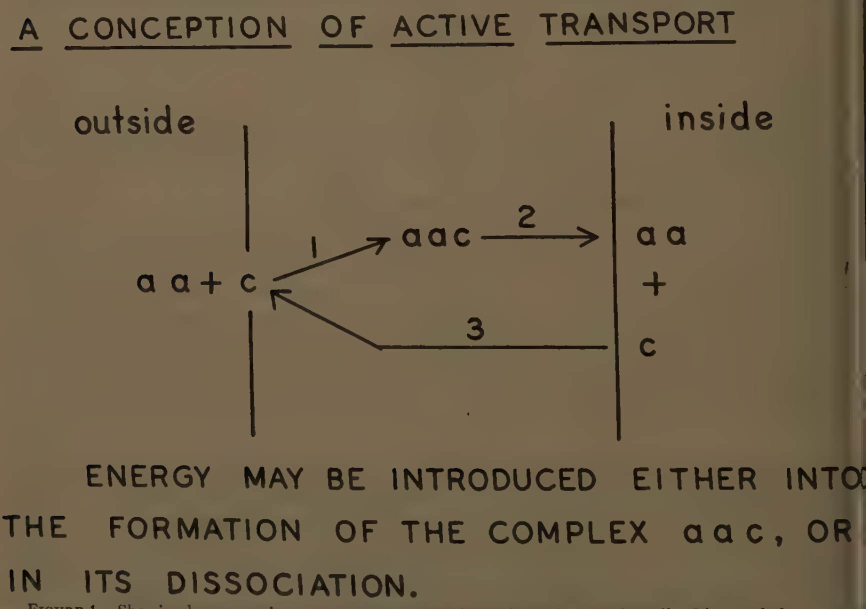
A CONCEPTION OF ACTIVE TRANSPORT
outside
inside
a a + c
1
a a c
2
a a
+
c
3
ENERGY MAY BE INTRODUCED EITHER INTO
THE FORMATION OF THE COMPLEX a a c, OR
IN ITS DISSOCIATION.

FIGURE 1. Showing how a carrier may operate to transfer amino acid into the cell. The symbol, aa, represents an amino acid.

carrier is visualized as acting in a cyclic fashion. The process illustrated here could transfer amino acids, but it would not concentrate them. To produce concentration, energy must be introduced into the process. There are a number of ways in which this might be achieved. In general, the carrier needs to be presented at a higher chemical potential or in a higher-energy form at the outside than at the inside of the membrane.

Let us turn now to the question of the nature of the acceptor or carrier to which the amino acids become bound during their transfer into the cells.

Riggs, in our group, noticed that the addition of pyridoxal intensified the degree to which amino acids were concentrated. He then produced a vitamin B_6-deficiency in mice, and showed that tumor cells from these mice were deficient in their ability to concentrate glycine.[8, 9] This effect has now been shown for a number of amino acids.

Phosphorylation of pyridoxal is probably not involved in this action, because 5-deoxypyridoxal is also effective, as is also 4-nitrosalicylaldehyde.[10] These analogs of pyridoxal have been shown by Ikawa and Snell to catalyze several reactions in the same way pyridoxal does.[11, 12]

Although the 5-methylol group of pyridoxal is not essential, the 3-phenolic hydroxyl group is necessary to this activity, as shown by the inactivity of 3-0-methyl pyridoxal. The aldehyde group is also essential; 4-deoxypyridoxine s ineffective.[9] Finally, the ring nitrogen or nitro group is necessary, since salicylaldehyde is inactive.[9]

This requirement for three structural features (aldehydic, hydroxyl, and nitrogenous groups) for amino acid transfer is explicable in terms of the type of complex visualized by Snell and his collaborators as operative in the cata-ytic action of these substances (FIGURE 2). These are metal-stabilized Schiff's bases of the amino acids.

There are a number of effects of amino acid structure upon their concentrative transfer which also support the involvement of such chelation reactions n the transfer process:

(1) Alpha- and beta-amino acids are concentrated[6, 13] but gamma-aminobutyric is not. Metal chelation occurs with the first two, but, for the last, the amino group and carboxyl group are too far apart for a stable chelate.

(2) Primary and secondary amino acids are concentrated but, in general, tertiary amino acids are not. N-methyl groups have been introduced into glycine, alanine, α-aminobutyric acid, α-aminoisobutyric acid, and proline to demonstrate this effect.[10] Were a simple metal chelation adequate, N-dimethylamino acids should be concentrated. These, for instance, form fairly stable copper salts. They cannot, however, form Schiff's bases, as can the primary and secondary amino acids. With the primary amino acids, aldimine and carbinolamine forms are possible, while the Schiff's base of the secondary amino acid is restricted to the carbinolamine form (FIGURE 2).

(3) The nickel salts of the primary and secondary amino acids react with pyridoxal to form chelates, as indicated by absorptimetric and titrimetric hanges.[10] With the tertiary amino acids, the acid-base titration and the bsorption of radiant energy were essentially unchanged from that calculated or the physical mixtures.

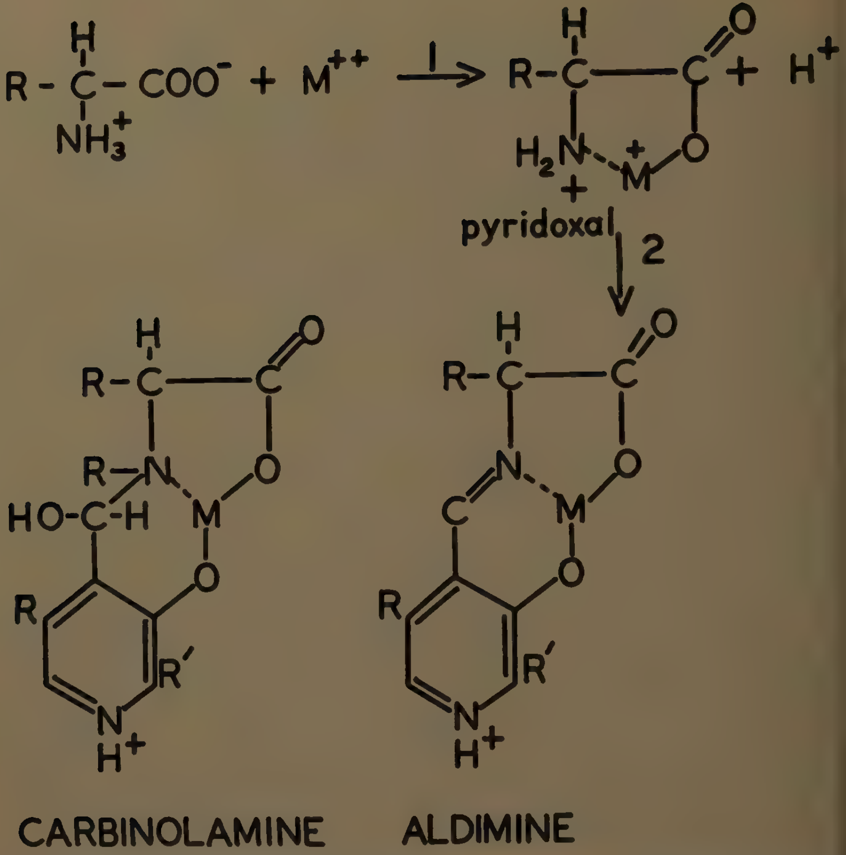

FIGURE 2. Chelation of an α-amino acid with a metal followed by Schiff's base formation with pyridox
The reaction may of course occur in the other order, in which case the displacement of the hydrogen ion is
tributed to the pyridoxal. The carbinolamine form only is possible with secondary amino acids.

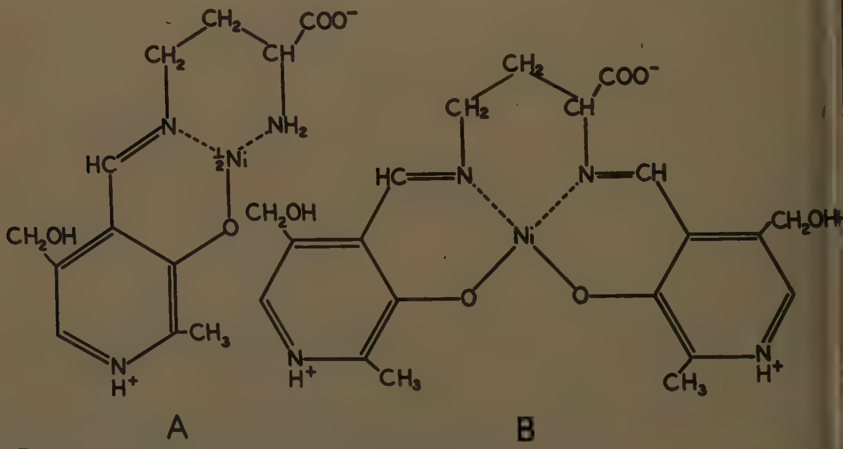

FIGURE 3. Possible chelates of α,γ-diaminobutyric acid with nickel and pyridoxal. In A, Schiff's b
formation may be to the α- rather than the γ-amino group. At higher pH the double Schiff's base B is conside
to predominate.

(4) A second nitrogenous group, two or three carbons removed from the ι-amino group, leads to a notable stimulation of concentrative uptake. α,γ-Di- minobutyric and α,β-diaminopropionic acids are so strongly concentrated as o cause major replacement of the cell potassium with the organic cation, and reat swelling.[14, 15] With ornithine and lysine, on the other hand, the uptake s modest. This remarkable sensitivity to the spacing between the two amino roups probably has a steric origin. It is proposed that the butyric and pro- ionic acid derivatives form chelates in which both amino groups are involved, situation already shown to apply to the copper salts of the amino acids.[16]

By Job's method of continuous variation,[17, 18] maximal absorption was ob- ained at pH 4.2 and 7.4 with the molar ratio 1:2:2 for nickel, pyridoxal, and iamino acid. At pH 10.4, however, much higher absorption was found with he ratio 1:2:1.[10] An absorption maximum at about 940 mμ was obtained in all ases. These results provide evidence that the most stable chelate at pH 7.4 as the composition, nickel (pyridoxal)$_2$ (diamino acid)$_2$, perhaps having the tructure A (FIGURE 3), but that at pH 10.4 the composition, nickel (pyridoxal)$_2$ iamino acid, predominates. The latter probably corresponds to Schiff's base ormation with *both* amino groups (formula B, FIGURE 3). The former com- osition seems the more likely in the living organism, especially considering he low pyridoxal levels.

The strong concentration of 2-pyridyl-DL-alanine[19] shows that highly active ptake may occur where Schiff's base formation is limited to the α-amino group, lthough metal-chelation can occur also to the γ-nitrogen. Whether this or he reverse order of linkage occurs in the transfer of α,γ-diaminobutyric acid as not been established.

In summary, the structural requirements, both on the part of aromatic alde- ydes for stimulation of transfer and on the part of the amino acids for highly ctive transfer, support the possibility that metal-chelated Schiff's bases of ne amino acids are formed during their concentrative transfer.

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AMINOPEPTIDASE CONTENT AS CORRELATED WITH CHROMOSOME CONSTITUTION IN SUBLINES OF THE EHRLICH ASCITES TUMOR*

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For some time we have been interested in the quantitative relation of peptidases to growth. Evidence has been accumulating that indicates that the peptidases are ubiquitous in cells and are present in highest concentration in those in which protein is rapidly synthesized, such as glandular cells or rapidly growing cells.[11] The precise relation of these proteolytic enzymes to protein synthesis is unknown, but the equilibrium of the proteolytic reaction is so far in the direction of split products that it is unlikely that the peptidases alone act synthetically. It is possible that they might act in conjunction with the systems involving the nucleic acids, or fragments thereof, substances that Gale and Folkes[2, 3] have shown to be necessary for the incorporation of certain amino acids into bacterial proteins.†

The ascites tumors offered an obvious opportunity to study the behavior of peptidases in a rapidly growing cell population where it was possible to measure enzyme content on a per cell basis. In addition, the derivation by Kaziwara[8] of exactly hypertetraploid sublines of the hyperdiploid Lettré-Ehrlich ascites carcinoma provided a material which seemed ideal for a quantitative study of the relation of chromosome number to given enzyme system in tumor tissue. The mode of the chromosome number of the derived polyploid sublines (EL88) is 90 to 92, whereas that of the original Heidelberg Ehrlich ascites is 45 to 46. Therefore, it appeared that the chromosome constitution of these sublines was far better defined than that of most tumors. These relatively blood-free ascites tumors furnished in abundance a countable number of viable cells that initially gave promise of a high degree of uniformity and reproducibility—a necessity for biochemical research on the cellular level.

The following is an account of our experience in working with the Lettré (LE) and EL88 sublines of the Ehrlich ascites carcinoma.‡ As will be seen the interpretation of the chemical data in cellular terms was not uncomplicated, but the results clearly showed a quantitative relation between chromosome content, cell size, nitrogen content, and that of the particular enzyme studied, labile aminopeptidases.

Materials and Methods

Five hypertetraploid sublines of the Lettré-Ehrlich ascites carcinoma were studied. The first, EL88-1, was derived by Kaziwara.[8] The second, EL88-2

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† The recent report of Binkley, Olson, and Torres (Abstra. Am. Chem. Soc. 36C, Minneapolis, Minn. September 1955) that aminopeptidases consist of nucleoside diphosphate glucosamine peptide complexes offers the possibility of a quite direct relationship.

‡ We are greatly indebted to Doctor Hauschka and to his assistants, Barbara Kvedar, David Hungerfor and Sally Grinnell, not only for providing the ascites tumors, but also for allowing us to use their facilities f transfer, and for teaching us the techniques involved.

vas taken from a sample of this tumor frozen by Kaziwara. The third and ourth and fifth sublines were obtained by the method of low cell dosages[8] from he second and third sublines, respectively, by Hauschka (EL88-3) and Podber EL88-4 and EL88-5). These sublines and the Lettré-Ehrlich (LE) hyper-liploid ascites were maintained by serial intraperitoneal transplantation into dult (1.5- to 4-month) female mice of the Swiss/Ha ICR strain. Tumor sam-les were taken at 7 to 10 days' growth, that is, at the asymptotic portion of he growth curve.[10]

As the work progressed, it was found necessary to carry out a steadily in-reasing number of cytological and histological control studies in order to give neaning to the biochemical data. Initially, it was thought that total cell ounts would suffice. These were done in a 1:275 dilution in M/60 phosphate uffered (pH 7.4) 30 per cent glycerine or 0.25 *M* sucrose solution. Later, o provide more information, the cells in the counting chamber were differenti-ted into large, small, and red cell categories. For better differentiation of umor from nontumor cells, acetic orcein-fast green smears were used. Rough netaphase chromosome counts* and counts of dead cells (in 0.25 *M* sucrose) vere made on these preparations. Finally, a more inclusive index of ploidy n the cell population was furnished by cytophotometric measurement of eulgen staining of the interphase cells by Doctor Jerome Freed. These neasurements were made on nuclei isolated in citric acid.

Volumes of the living cells and nuclei were calculated as spheres from meas-rements of diameters in a Petroff-Hausser counting chamber, using phase ptics and a net ocular micrometer. These cell suspensions were diluted with scites serum.

The peptidase determinations were carried out using Linderstrøm-Lang and Iolter ultramicrochemical methods.[11] The 15 μl. volume used in the reaction nixture allowed a considerable saving on substrates without loss in final ac-uracy. The dipeptide and tripeptide substrates were all in the L-form except lanylglycine, which was DL. Final substrate concentrations were 0.040 to .045 molar, made up to pH 8.0 (25°) (about pH 7.6 at 40° C.) with sodium hy-roxide. The substrates were used for buffering, as all reactions were stopped efore 50 per cent hydrolysis. The time for the reactions at 40° C. was one our in most cases, longer times being necessary for the preliminary experi-nents on substrates whose rate of hydrolysis was relatively low. The reactions vere zero order, hydrolysis being proportional to time and enzyme concentra-ion. Activities were calculated on a per cell basis in terms of values obtained rom acid titration of amino groups in acetone and are expressed as microliters f N/20 HCl per hour per cell. Reagent and enzyme blanks and determina-ions were simultaneously run in triplicate. In general, the values agreed vithin 5 per cent, and the enzyme blank values were no greater than 10 per ent of the value of the determinations.

Aliquots of two types of tissue preparation were used: (1) whole cells re-eatedly frozen and thawed in 30 per cent glycerine, M/60 phosphate buffer

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* We are indebted to Doctor Hauschka and to others, especially D. A. Hungerford, for detailed metaphase udies on several samples of the EL88 sublines.

Table 1

Relative Rates of Peptide Hydrolysis by Cell Suspensions

Substrate	Ratio of activities (LA–Mn = 1.0)		
	Drosophila salivary gland	Mouse ascites tumor	
		LE	EL88-1
Alanylglycine	22	17	15
Glycylleucine	18	18	15
Leucylglycine	10	3	3
Leucylglycylglycine	2	3	3
Glycylglycylglycine	2	1	1
Leucineamide-MnCl$_2$	1	1	1

(pH 7.4); and (2) homogenates in phosphate buffered 0.25 *M* sucrose. Fiv minutes homogenization in a Servall omnimixer at full speed was required fo disruption of the majority of the ascites cells, which had previously been washe twice with isotonic sucrose. The peptidase activity of these homogenat was not lowered by storage at −40° C.

Various other chemical analyses were carried out on aliquots of the froze homogenates. Total nitrogen was determined by a micromodification (fin volume 2 ml.) of the Kjeldahl and Nesslerization procedures.[18] Analyses fo total nucleic acid, PNA, and DNA were carried out by our ultramicromodifi cation of the Schneider method[16] using perchloric acid. The total nucleic acid calculated from the sugar reactions (orcinol and diphenylamine) checked close with those calculated from U-V absorption or phosphorus analyses. Duplica extractions agreed within an average of 5 per cent.

Results

Preliminary experiments* using whole cell suspensions (TABLE 1) showe that the relative rates of hydrolysis of the various peptides by these ascit tumors were, in general, similar to each other and also to those previous found in other tissue such as the salivary gland of *Drosophila melanogaste* The exceedingly labile peptidases splitting alanylglycine (AG) and glycylleuci (GL) were the most active of those studied, showing 17 to 18 times the activi of the stable leucine aminopeptidase. Glycylleucine was chosen as substra for comparative experiments on the hyperdiploid and hypertetraploid subline Addition of metal ions to the system resulted in no activation, suggesting th the tissue provided optimal metal concentrations.

It soon became apparent that higher peptidase values were obtained homogenates than in suspensions of intact cells, where only partial cytoly had occurred after repeated freezing and thawing. It seemed that adequa substrate-enzyme contact had not been achieved in these preparations an therefore, all subsequent work was carried out on homogenates. Check e periments on homogenates gave the same ratios between substrates as tho recorded in TABLE 1 for cell suspensions.

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* We wish to acknowledge the excellent technical assistance of Helga Lang in these experiments.

TABLE 2

VARIABILITY OF CELL POPULATION IN SUBLINES OF EHRLICH ASCITES TUMORS IN ICR SWISS MICE

Subline	Date	Days growth	Cells per 0.1 µl. (1:275)	Per cent		
				R.b.c.	Nontumor	Hypertetraploid metaphases
EL88-1	4/29	10	54.0			91*
	5/7	8	49.6			
	5/17	10	35.5		18.1	93
	5/24	9	46.3		24.7	90
	10/7	10	59.7		8.0	50*
EL88-2	10/28	8	58.0	5.7	12.4	93*
	11/5	8	40.4	13.3	26.3	88
	11/13	8	50.3	7.3	12.7	73
	11/22	9	61.6	0.8	11.1	47
EL88-3	12/10	10	41.7	2.0	8.2	98*
	12/20	10	52.3	5.4	12.9	98
EL88	Means		49.90	5.75	14.10	
LE	Means range		86.07 48.4–118.8	2.63 1.2–4.8	10.21 5.1–15.4	

* We are indebted to Doctor Hauschka and his colleagues for these counts.

The conditions for enzyme assay being established, attention may be turned the results of histological and cytological studies of the sublines and their fect on quantitative comparisons of these lines. As seen from TABLE 2, the umber of cells per unit volume of the EL88 ascites is a little over half that in e Lettré-Ehrlich as previously determined by Hauschka and Grinnell.[5] The d cell contamination was slightly greater in the tetraploid tumor than in the ploid. In both cases, the amount of peptidase contributed by the red cells as within the experimental error. Therefore, red cells were omitted from e cell counts in the later experiments on homogenates, which introduces an ror in the nitrogen determinations, but not in the DNA calculations.

The varying percentages of nontumor cells posed a problem for the peptidase r cell calculations, the mean values (14.1 per cent) for the EL88 sublines ing slightly higher than the LE (10.2 per cent). An attempt was made to lculate a mean peptidase per cell value for the nontumor cells by using simulneous equations. It was found, however, that the variability of the material as too great to make valid use of this mathematical method. Actually, by ch calculations, an average value was obtained for nontumor cells that apoximated the value for the LE cells. Assuming this value, the contaminaon with nontumor cells would not affect the peptidase per LE ascites cell. the case of the hypertetraploid tumor, however, the mean value per cell, cluding the relatively high percentage of nontumor cells, would be lowered d, hence, the observed EL88 and LE peptidase ratio would be lower than e true value. Similarly, the absolute value of the EL88/LE ratio for nitrogen content would be lowered by admixture of small nontumor cells of lesse nitrogen content than that of the tumor cells. Counts showed that dead cell in the sucrose preparations averaged 3 to 6 per cent.

A far more serious problem, however, concerns the ploidy of the ascites cell themselves. As has been pointed out by Kaziwara,[8] many of the hypertetraploid sublines show an increasing proportion of hyperdiploid cells in succeedin transplant generations. This complicated our experiments considerably. A 10/7, metaphase counts revealed that the Kaziwara EL88-1 subline was onl 50 per cent hypertetraploid in contrast to the 90 to 93 per cent five month before. The subline EL88-2, re-established by Hauschka from a frozen sampl of the EL88-1, again reverted to 50 per cent tetraploid after three serial transfers. Using a low dosage re-extraction, however, Doctor Hauschka was abl to obtain a 98 per cent tetraploid (EL88-3), but this also showed about 5 per cent hyperdiploid metaphases by the ninth generation. Nevertheless, th quantitative chemical determinations made on this subline gave results worth of detailed report (see TABLES 3 and 4). It might also be noted that the las subline studied, EL88-5, had reverted to 56 per cent tetraploid after four transfer generations.

Comparison of the peptidase and nitrogen values with the metaphase count (TABLE 3) showed a disconcerting lack of correlation between the chemic and cytological data. It might be expected that, as the ascites became mo diploid, the chemical values would approach the Lettré subline values. N such trend was observed, and the coefficient of variability of the mean value for peptidase and nitrogen in the EL88-3 subline, while higher than those f the LE subline, were not excessive considering the probable errors in cell coun ing.[4] Doctor Jack Schultz suggested that the discrepancy might be due t the presence in the EL88 subline of interphase cells of higher ploidy that ha

TABLE 3

GL-PEPTIDASE AND NITROGEN PER CELL IN SUBLINES EL88-3 AND LE

Subline	Generation	Hypertetraploid metaphases	GL-peptidase	Nitrogen
		%	μl HCl × 10^{-4}/hr.	$\mu\mu$g.
EL88-3	IV	93	6.53	56.8
	V		7.42	59.2
	VI	75	7.20	62.5
	VII	80	8.22	50.5
	VIII		7.85	63.4
	VIII	60*	6.39	46.1
	IX		7.73	65.8
	IX	49	8.15	63.7
	Mean		7.44 ± 0.25	58.5 ± 2.5
	coef. var.		9.3%	11.9%
LE	Mean		4.20 ± 0.10	34.0 ± 0.5
	n		6	7
	coef. var.		5.3%	3.7%

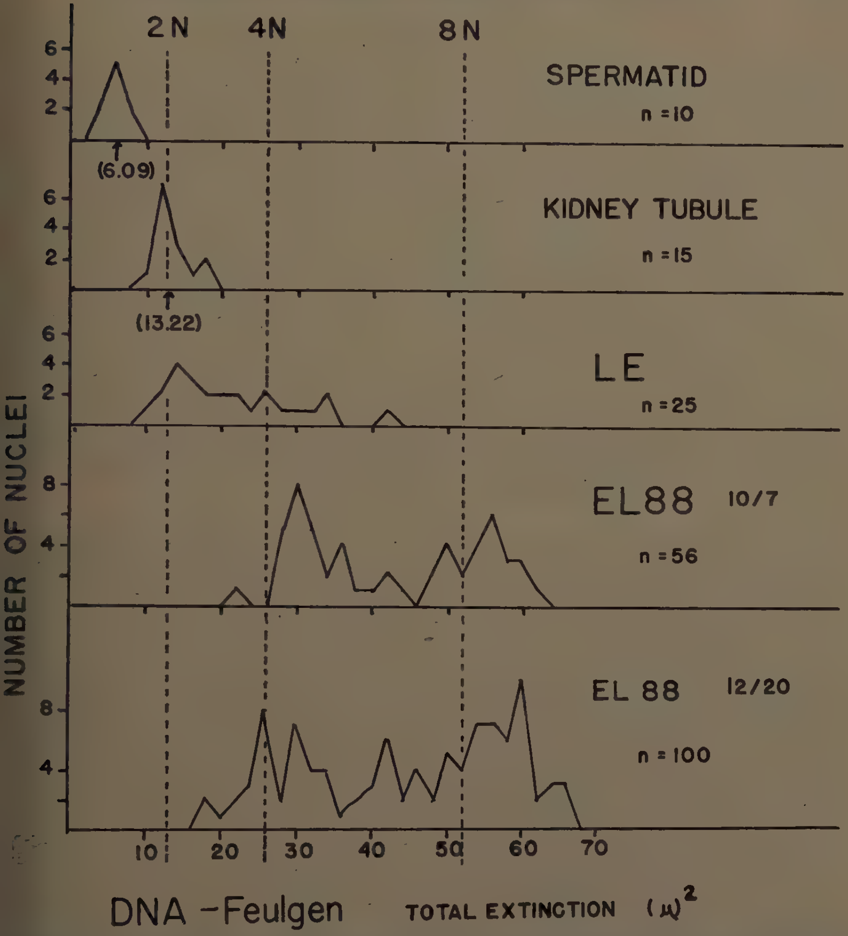

FIGURE 1. Frequency distribution of total extinction values (DNA-Feulgen) on nuclei from mouse normal ssues and ascites tumors (data from Doctor Jerome Freed). The total number of nuclei measured are shown y the *N* values. The 10/7 EL88 ascites was 50 per cent hypertetraploid by metaphase counts, the 12/20 sample, per cent hypertetraploid. The dotted vertical lines indicate ploidy values derived from extinctions measured kidney tubules, assuming the latter to be 2 *N*. The spermatid (1 *N*) value is given for comparison.

esulted from endoreduplication. These cells would not be represented in the ietaphase counts. The investigation of this possibility was undertaken by octor Jerome Freed, whose data are presented in FIGURE 1. The reference issues, mouse kidney, and spermatids were in the expected 2:1 ratio. The yperdiploid LE subline showed a distribution of DNA values reasonable for n actively growing ascites including about 7 per cent tetraploid cells. But he EL88 ascites contained many nuclei with ploidy values 8 *N* and over. he almost identical distribution of the two EL88 samples was surprising, as he 10/7 was only 50 per cent hypertetraploid by metaphase chromosome counts nd the 12/20 sample 98 per cent hypertetraploid. Thus, the assumption of he presence of interphase cells of high ploidy was confirmed, and an explana-

TABLE 4

DNA PER CELL IN SUBLINES EL88-3 AND LE

Subline	Generation	Date	DNA per tumor cell	Ploidy calc. from DNA	Ploidy calc. from Feulgen	Hypertetra ploid metaphase
			μμg.	N	N	%
EL88-3	III	12/20	(22.5)	6.6	7.5	98*
	IV	1/4	16.3	4.8		93
	V	1/13	21.7	6.4		
	VI	1/21	22.4	6.6		75
	VII	1/31	17.0	5.0		80
	VIII	2/7	17.6	5.2	5.1	
	VIII	2/7	13.6	4.0		60*
	IX	2/14	18.5	5.4		
	IX	2/14	13.0	3.8		49
	Mean of 8		17.5 ± 1.5			
LE	Mean of 5		10.0 ± 0.3	2.9	3.2	

* We are indebted to Doctor Hauschka and his colleagues for these counts.

tion for the relatively constant peptidase and nitrogen values was obtaine It was therefore deemed necessary to obtain DNA values by chemical dete minations on aliquots of all the frozen samples of the homogenates used f peptidase and nitrogen analyses.

The data from the chemical determinations of DNA are given in TABLE These DNA values are corrected for the DNA content of the nontumor cell assuming an average diploid DNA value of 6.8 μμg. per cell, following E Klein.[9] The ploidy values in the next column were obtained by dividing th DNA values by 3.4. Comparison of these figures with average values calc lated from the Feulgen-DNA analyses shows a reasonable agreement betwe the two techniques. Closer inspection of the DNA data shows that the mous to-mouse variability was almost as great as the differences observed betwe the values for samples 98 per cent tetraploid by metaphase count and tho 49 per cent. There was also a variability between sublines, as EL88-4 an EL88-5, when both, containing 92 to 97 per cent hypertetraploid metaphase gave average DNA per-cell values of 15.1 ± 6 and 17 μμg. respectively. It evident from these DNA determinations and from the Feulgen-DNA analys that metaphase chromosome counts in these instances gave an index of t state of ploidy of only a fraction of the cell population.

Pentose nucleic acid (PNA) analyses were made on all the samples, and was found that the frozen homogenates gave slightly lower values per c than the fresh cell suspensions. This was attributed to some breakdown PNA during the preparation and storage of the homogenates. For this reas valid comparison between the sublines could not be made, and the PNA da are not included in the tables.

Comparison of the sublines EL88-3 and LE, with respect to GL peptida nitrogen, volume, and DNA, is given in TABLE 5. It should be noted that t EL88 cell volumes were derived from samples showing 80 to 98 per cent hyp tetraploid metaphases, whereas the peptidase and nitrogen and DNA data i

TABLE 5

COMPARISON OF THE EL88-3 AND LE SUBLINES

Subline		GL-peptidase	Nitrogen	Volume		DNA
				Cells	Nuclei	
		μl. HCl × 10^{-4}/hr	μ μg.	μ^3	μ^3	μ μg.
EL88-3	Mean	7.44 ± 0.25	58.5 ± 2.5	2026	457	17.5 ± 1.5
	n	8	8	88	60	8
LE	Mean	4.20 ± 0.10	34.0 ± 0.5	1099	260	10.0 ± 0.3
	n	6	7	101	40	5
$\frac{\text{EL88}}{\text{LE}}$	ratio	1.77	1.72	1.84	1.76	1.75

uded samples of only 50 to 60 per cent. Considering the various errors ivolved, the agreement in the EL88/LE ratios is surprisingly good. There re obviously no significant differences between these ratios.

Discussion

The result that is apparent from these data is that in these tumor cells, the ıbile aminopeptidases are synthesized at a rate proportional to the growth of ıe cell. Cell size is proportional to chromosome content, as measured by NA. In addition, the relative amounts of the peptidases hydrolyzing the arious substrates studied are not changed as a result of the doubled chromo- ome numbers. Whether a more exhaustive analysis of a less limited group f peptide substrates would show differences remains an interesting problem.

Comparison of these results on peptidases with those obtained on other cells ıows that the ascites populations behave like those where growth takes place y interphase synthesis. Ogur[14, 15] has found that in a polyploid series of easts, respiration and fermentation per cell, as well as the dry weight and verage cellular content of nucleic acids, are exactly proportional to the ploidy f the cells. The situation, however, is different in cases where synthesis and orage of cell constitutents has taken place prior to a series of successively ıpid cell division as in the cleavage of eggs[6, 7] and division of some protozoa.[1] hen, the alanylglycine peptidase content is equally distributed between the aughter cells, and the relationship to the chromosome complement changes. special case exists in the salivary glands of giant larvae of *Drosophila*, where owth takes place by increase in cell size only, and the peptidase content in- eases along with cell volume and nitrogen content, although the DNA con- nt remains constant.[17] In all of the above types of growth, the peptidase ontent remains proportional to that of the active protoplasm as pointed out any years ago by Linderstrøm-Lang and Holter.[12] It would seem, then, ıat in the case of the peptidases, as in the other biochemical systems that ıve been studied, the Ehrlich ascites tumor cell shows no unique properties.

Summary

(1) Aminopeptidase content was studied in relation to chromosome consti- ıtion in the hyperdiploid Lettré-Ehrlich (I E) mouse ascites carcinoma and

in the hypertetraploid sublines (EL88) originally derived from this by Kaz wara.

(2) The chromosome constitution of these cells was determined by thre methods. Data from metaphase chromosome counts indicated that, in th successive transplant generations studied, the hypertetraploid sublines pro gressively regressed towards a hyperdiploid condition. But cytophotometri Feulgen-DNA analyses and chemical determinations of DNA per average ce showed that the DNA content of the interphase cells remained high. There fore, it appeared that, in this material, chromosome counts gave an index the state of ploidy of only a fraction of the cell population.

(3) Analyses of the sublines were made for content per cell of glycylleucin peptidase and DNA, and, in addition, measurements of total nitrogen and ce and nuclear volumes were used for reference. The values in each case gav a ratio of EL88 to LE of about 1.75. The content of this aminopeptidas therefore, had a positive correlation with cell volume, nitrogen content, an chromosome constitution as measured by DNA content in hypertetraploid an hyperdiploid sublines of Ehrlich ascites tumors.

(4) Enzyme assays were carried out with several other typical peptide These substrates were hydrolyzed in the following order of descending rate glycylleucine, alanylglycine, leucylglycine, leucylglycylglycine, triglycine, an leucineamide. The relative rates of peptide hydrolysis were similar, regardle of the chromosome content of the ascites subline, and they also closely approx mated those previously reported for some other biological materials.

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Discussion of the Paper

Doctor Mary E. Maver (*Enzymes and Metabolism Section, Laboratory of iochemistry, National Institutes of Health, Bethesda, Md.*): This work is of speial interest to those who are studying the protein metabolism of tumor growth, ince it has often been suggested that the chromosomes control the duplication f the specific proteins that are characteristic of a cell. The ascites tumors eem to be well suited to these studies.

In this report, the increase in a specific peptidase activity—together with ncreased protein and cell size—was correlated quantitatively with the inreased number of chromosomes.

In view of the immunological differences reported by Hauschka and others n the polyploid ascites tumors, it would be interesting to know if any qualitaive changes have occurred in the proteins of these tetraploid cells. Our techiques in protein-enzyme chemistry have not yet advanced to the stage where uch a qualitative analysis is possible.

As Doctor Patterson has said, the precise relation of the proteolytic enzymes

Table 1

Average Values of Catheptic Activity of Rat Hepatic Nuclei*

umber experiments	Tissue	Total N per ml. nuclei	DNA mg./mg. N	PNA mg./mg. N	Hydrolysis	
					Tyrosine	Soluble N
5	Normal liver	0.246	0.380	0.163	0	0.016
3	Regenerating liver 48 hrs.	0.312	0.375	0.233	0	0.164
4	Regenerating liver 72 hrs.	0.198	0.273	0.246	0	0.242
3	Regenerating liver 120 hrs.	0.211	0.277	0.190	0	0.090
5	Hepatoma 3683 (di-AAF)	0.436	0.818	0.227	0.103	0.179
3	Hepatoma 3924C (di-AAF)	0.328	0.924	0.312	0.133	0.191
4	Hepatoma N (DAB)	0.221	0.497	0.407	0	0.121

* Summary table in catheptic activities of nuclei of normal, regenerating, and neoplastic tissues of the rat. . E. Maver, A. E. Greco, E. Løvtrup, and A. J. Dalton. 1952. J. Natl. Cancer Inst. **13:** 687–703.

The average total N in mg. per ml. suspension of nuclei is given. The catheptic activities were obtained by termining the mg. tyrosine and TCA soluble N liberated from hemoglobin in 1 hour per mg. N in the suspenon of nuclei.

(di-AAF)—Hepatoma 3683 and adenocarcinoma 3924C originally induced by feeding 2-diacetylaminofluorene.

(DBA)—Hepatoma N, originally induced by feeding *p*-dimethvlaminoazobenzene (butter yellow).

to protein synthesis is unknown, and the probable role of the nucleic acids obscure at this time. Numerous reports in the literature, however, suppo the view that these factors may be interrelated and may function in growth

In a study with Greco, Løvtrup, and Dalton, we have determined the ac tivity of the intracellular proteinase, cathepsin, in the isolated nuclei of hepat cells whose normal rate of growth had been stimulated either by a natural r generative process or by carcinogenic agents. Using a modification of th procedure described by Wilbur and Anderson, the nuclei were separated b differential centrifugation in layered sucrose-calcium chloride solutions. Th relative values for catheptic activities, DNA and PNA, were based upon th nitrogen content (TABLE 1). It should be noted here that the catheptic ac tivity is concentrated in the mitochondria of cells. Normal liver nuclei neede an hour's digestion period to show any digestion of hemoglobin.

With the regenerating livers, the largest nuclei, as seen with the phase-co trast microscope, were obtained 72 hours after partial hepatectomy when th catheptic activity, according to the TCA soluble N figures, was the highes These nuclei gave the highest soluble N values of any of the hepatic nucle while the DNA/N had decreased from the normal value. Five days after th partial hepatectomy, the nuclei had almost returned to normal size and th catheptic activity was definitely decreased.

The neoplastic nuclei showed a closer correlation between the size, cathept activity, and DNA/N content than the normal nuclei. The di-AAF tum nuclei were greatly enlarged and had multiple large nucleoli. These morph logic changes were accompanied by more than a twofold increase in DNA/ and a tenfold increase in catheptic activity over that of the values for norm liver nuclei. The morphologic changes in the nuclei of the DAB induced hepa toma were slight, and their average diameter was about half that of the nucl of the di-AAF neoplasms. This corresponded to the DNA/N values and th lower catheptic activities of these nuclei.

The absence of tyrosine from some of the digests raises the question of possib qualitative changes in the enzyme systems of the nuclei.

I have presented some results of attempts to correlate size and protein sy thesis with only one proteolytic enzyme. Many other enzymes, particular the energy-yielding oxidative enzyme systems, as well as the nucleases, a probably involved in this very complex growth phenomenon.

STUDIES OF NUCLEIC ACID SYNTHESIS IN ASCITES TUMOR CELLS[*]

By G. A. LePage, J. Greenlees, and J. F. Fernandes[†]
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Introduction

It has been established that ascites cell tumors can be used with facility for *i vitro* studies of nucleic acid purine synthesis.[1] The standard conditions sed involved anaerobic atmosphere (95 per cent N_2-5 per cent CO_2), a glu- ose-salts medium with bicarbonate buffer at pH 7.4 and incubation at 38° C. ı oversize Warburg respirometer vessels. When glycine-2-C^{14} was added to ıis medium, it was incorporated into the purines of both RNA and DNA. 'arallel studies have been made *in vivo* by intraperitoneal injection of the lycine-2-C^{14} and other factors under study, in ascites-cell-bearing mice.

Azaserine has been found to be a very potent inhibitor of these two biological est systems, as it has in at least two other laboratories studying purine syn- ıesis.[2, 3]

Experimental

The extent to which incorporation of glycine-2-C^{14} into various components f ascites tumors occurs is illustrated in TABLE 1. Approximately 15 per cent f the radioactivity of the added glycine-2-C^{14} is present in the proteins after one-hour incubation.

In TABLE 2 are data concerning the effects of added azaserine on the incor- oration of glycine-2-C^{14} into the various cell components. Protein was found ɔ be unaffected. The incorporations into RNA purines and DNA purines ere affected approximately equally. Data on nucleic acid purines (NA) in ıter tables are for mixed nucleic acids obtained by extraction with hot per- hloric acid. The acid-soluble adenine fraction (AS) contains the adenosine ıonophosphates, diphosphates, triphosphates, and other coenzymes.

TABLE 3 gives data for the *in vitro* inhibition of three different ascites cell ımors by azaserine. It can be seen that 0.10 μg. of azaserine is sufficient to ive almost complete inhibition and that, at 0.05 μg., the inhibition drops. .ny increase above 0.10 μg. gives the same result. There appears to be a small vel of incorporation even at much higher levels of the inhibitor (4 to 8 per ent of the control value).

For *in vivo* studies, the azaserine was injected intraperitoneally in ascites ımor-bearing mice. After the appropriate time, glycine-2-C^{14} was injected ıtraperitoneally at 100 μg. per 25 gm. mouse (18 × 10^6 cts./min./mg.–4.0 ıg./kg. body wt.) and allowed one hour to incubate. Then the cells were ithdrawn and inactivated with perchloric acid. The peak glycine incorpora- on occurs after two to three hours. Hence this arbitrarily chosen time is on

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[†] Rockefeller postdoctoral fellow, on leave from the University of Sao Paulo, Brazil.

TABLE 1

IN VITRO INCORPORATION OF GLYCINE-2-C^{14} INTO EHRLICH ASCITES TUMOR CELLS

Cts./min./mg.

Acid-soluble adenine	RNA		DNA		Protein
	Adenine	Guanine	Adenine	Guanine	
130,000	7500	9000	4200	4500	6500

150 mg. wet weight of cells in 12 ml. of salts-glucose medium under anaerobic conditions—incubated 1 hour 38°C. with 150 µg. glycine-2-C^{14} (18 × 10^6 cts./min./mg.).

TABLE 2

EFFECTS OF AZASERINE ON THE IN VITRO INCORPORATION OF GLYCINE-2-C^{14} INTO EHRLICH ASCITES TUMOR CELLS

	Per cent inhibition	
	Adenine	Guanine
Acid-soluble	52	—
RNA	71	64
DNA	58	48
Protein	0	

150 mg. wet weight of cells in 12 ml. of salts-glucose medium under anaerobic conditions—incubated 1 hou 38°C. with 150 µg. glycine-2-C^{14}, with and without addition of 0.05 µg. azaserine.

TABLE 3

IN VITRO INCORPORATION OF GLYCINE-2-C^{14} % INHIBITION BY AZASERINE

Tumor	Addition	AS adenine	NA adenine
	µg.		
Ehrlich	0.05	52	67
	0.10	96	92
TA 3	0.10	93	92
S180	0.10	95	92

TABLE 4

IN VIVO INHIBITION OF GLYCINE-2-C^{14} INCORPORATION (%)

Tumor	Addition	AS adenine	NA adenine
	µg.		
Ehrlich	1	44	47
Ehrlich	5	99	100
S180	1	97	97
S180	5	92	92

The azaserine was injected intraperitoneally into 25 gm. mice and 3 hours later glycine-2-C^{14} was given incubated 1 hour.

TABLE 5

DURATION IN VIVO OF AZASERINE INHIBITION (%) ON INCORPORATION OF GLYCINE-2-C^{14}

Tissue	Injection	Time (hours)	AS adenine	NA adenine
	μg.			
hrlich	10	10	94	97
hrlich	10	24	63	40
hrlich	5	12	86	80
hrlich	5	24	71	41
pleen	10	10	43	30
pleen	10	24	28	29
pleen	5	12	0	0

Azaserine injected intraperitoneally in 25 gm. mice. After the indicated time, glycine-2-C^{14} was injected nd incubated 1 hour.

TABLE 6

EFFECT OF AZASERINE ON AVERAGE SURVIVAL TIME (DAYS) OF ASCITES-CELL TUMOR-BEARING MICE

Tumor	Controls	Azaserine-treated	
		1× daily	2× daily
A3	16 (13)*	18 (13)	25 (8)
hrlich	15 (6)	18 (8)	21 (8)
C3HED	13 (10)	17 (10)	22 (10)
180	8 (10)	13 (10)	18 (10)
A3	16 (10)		32† (10)

* No. of mice per group.
† Treated 12 days instead of 6 days.

TABLE 7

EFFECTS OF AZASERINE ON THE IN VITRO INCORPORATION OF GLYCINE-2-C^{14} INTO PURINES OF EHRLICH ASCITES CELLS WITH VARIED LEVELS OF TISSUE

Tissue-mg. dry wt.	Per cent inhibition	
	NA adenine	NA guanine
15	79	30
30	67	34
60	79	55

Incubated anaerobically at 38°C. with 150 μg. glycine-2-C^{14}, with and without addition of 0.05 μg. azaserine.

e rising part of the curve. TABLE 4 provides some data concerning the *in vo* inhibition caused by azaserine. At the lower dose, 0.04 mg./kg., Ehrlich seen to be less susceptible than Sarcoma 180. However, both are almost mpletely inhibited by 0.2 mg./kg.

Experiments were conducted to determine the length of time this inhibition mained in effect *in vivo*. Some data from such experiments are shown in BLE 5. Here the results with spleen are included to illustrate the effects n a "normal" growing tissue. The spleens of these animals enlarge several

TABLE 8

EFFECT OF WASHING THE EHRLICH ASCITES CELLS FROM AZASERINE TREATED MICE ON IN VITRO INHIBITION OF GLYCINE-2-C^{14} INCORPORATION

Tissue	Cts./min./mg.	
	NA adenine	NA guanine
Washed control cells	4000	6500
Washed control cells + azaserine	0	700
Pretreated cells*	0	500
Pretreated cells + azaserine	0	400

* Cells were withdrawn from Ehrlich ascites-bearing mice and 10 μg azaserine given intraperitoneally p
mouse. After two hours the remaining cells were withdrawn and both cell populations washed four times b
suspension in seven volumes of saline each time.

fold during the growth of the tumors. As illustrated in this table, the inhib
tion is almost complete for 12 hours with a dose of either 0.2 mg. or 0.4 mg./kg
body weight. By 24 hours, it has "worn off" to a considerable extent. Th
spleen was affected much less than the tumor cells at the higher dose, and no
at all at the lower dose.

To determine the relationship between the *in vivo* biochemical measuremen
and observed survival of the animals, experiments were conducted in whic
mice of the appropriate strain were injected intraperitoneally with 8 × 10
ascites cells each. After 24 hours, three groups were set up for each tumo
a control group that was injected once a day with saline, a group injected intra
peritoneally once a day for six days with 0.4 mg. azaserine/kg. body weigh
and a group injected twice a day at seven- to eight-hour intervals for six day
with 0.2 mg./kg. body weight at each injection. After the six-day treatmen
the animals were not treated further, and survival time was observed. A
additional experiment involving 12-day treatment is also included. The in
creased survival time on treatment with azaserine was observed to correla
well with the time that biochemical inhibition would be in effect. The resul
are shown in TABLE 6.

There were several unusual features to the observations on *in vitro* actio
of azaserine. One of these is illustrated by data presented in TABLE 7. At t
dose of azaserine that did not completely inhibit the system, the level of tissu
was varied. The extent of inhibition does not appear to be proportional t

TABLE 9

EFFECTS OF AZASERINE ON THE IN VITRO INCORPORATION OF GLYCINE-2-C^{14} INTO EHRLICH ASCITES CELL PURINES WITH TIME

Incubation time min.	Per cent inhibition	
	NA adenine	NA guanine
15	48	0
30	75	32
60	92	61
30*	78	35

* Incubated with the azaserine (0.5 μg.) 15 minutes at 28°C. before the glycine-2-C^{14} was added.

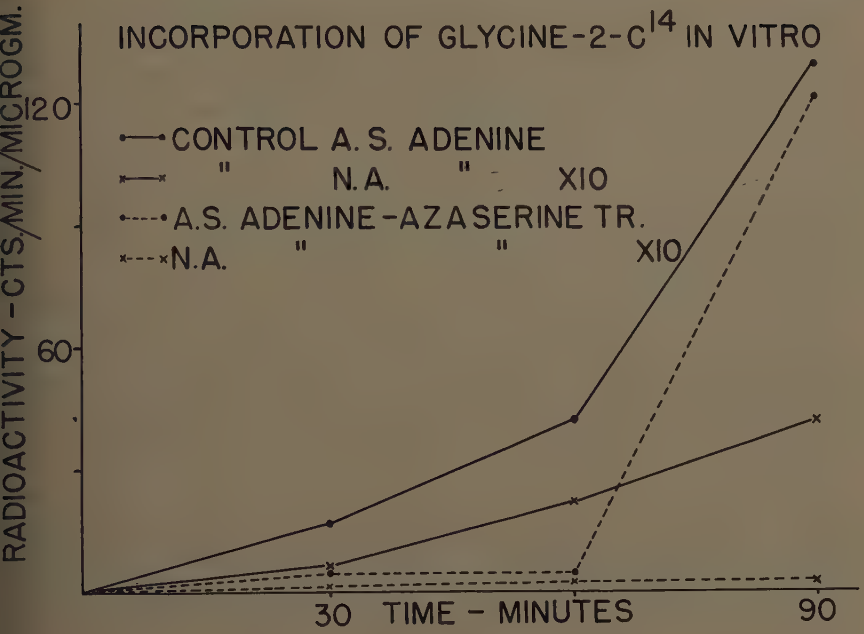

FIGURE 1

ssue concentration. However, since azaserine is quite soluble in water and ctive *in vivo* over a period of hours, it was reasonable to expect that it was ound to the cells. TABLE 8 provides data from an experiment in which azaerine was administered to the cells *in vivo*, and the cells withdrawn for an *vitro* experiment. Washing the cells by suspending them repeatedly in saline as been shown to remove endogenous glycine.[1] The data indicated, however, at azaserine was not washed away.

Another interesting characteristic of the *in vitro* effects of azaserine is shown TABLE 9. During the first few minutes of the incubation, the inhibitory ffect of azaserine is less, and it increases until almost complete. It is this arly incorporation of glycine-2-C^{14} which is the less susceptible 4 to 8 per cent

TABLE 10

EFFECTS OF SUPPLEMENTS TO THE MEDIUM ON THE INCORPORATION OF GLYCINE-2-C^{14} BY EHRLICH CELLS IN VITRO—90 MINUTES INCUBATION

Additions	AS adenine	NA adenine
	% Inhibition	
ontrol	96	92
lutamine	50	60
lutamine, ribose phos., CF, B_{12}, homocysteine, formate, NH_3	66	63
oiled juice*	0–35	0–35

* Made by heat-inactivating rat or mouse liver.

TABLE 11

EFFECTS OF SUPPLEMENTS TO THE MEDIUM ON THE INCORPORATION OF GLYCINE-2-C14 BY EHRLICH CELLS IN VITRO

Additions	AS adenine	NA adenine	NA guanine
	% Inhibition		
Control	100	95	89
+Glutamine, *etc.*	66	64	61
+Boiled juice (3 min. heat)	31	18	17
+Boiled juice (6 min. heat)	31	20	17
+Boiled juice (3 min. heat + acetone to 80%)	35	49	34

of the reaction observed in longer incubations. This would appear to indicat
the need for a measurable time in binding azaserine to a cell constituent, or i
converting it to an active form. However, incubation with the azaserine be
fore the addition of glycine-2-C^{14} did not give a result that would support thi
interpretation.

In running time curves on the inhibition of the *in vitro* system by azaserine
a result was encountered that is illustrated in FIGURE 1. This result was ob
tained in 6 of about 20 experiments. With 0.5 μg., azaserine per reaction ves
sel, the inhibition of incorporation into AS adenine was at the usual level unt
60 minutes. Between 60 and 90 minutes, incorporation in azaserine-treate
vessels was at an accelerated rate and almost reached the control level. I
appeared that inhibition was overcome, possibly as a result of accumulate
metabolic pools of intermediates. We attempted to bring this about in
more predictable manner by making additions to the suspending medium
Since Hartman *et al.*[2] have found that in their pigeon-liver system, azaserin
inhibition is overcome by the addition of glutamine, this was added to ou
system at 1 micromole per vessel. This gave only partial relief of the inhib
tion, as illustrated in TABLE 10. The addition of the other cofactors listed di
not further enhance the glutamine effect. The addition of extracts prepare
by heating mouse liver homogenate gave complete relief of the inhibition i
some cases, and was always superior to glutamine, except where glutamine wa
added at very high levels, which could not conceivably be present from th
liver extract (used extract from 100 mg. liver per vessel).

Since Reilly[4] has reported that liver is a good source for an enzyme tha
destroys azaserine by deamination, the tests described in TABLE 11 were con
ducted. It appears unlikely, from these data, that the relief of inhibition
due to destruction of the azaserine by liver enzyme.

Summary

Ascites tumor cells are useful tools for the study of nucleic acid purine syn
thesis *in vitro* and *in vivo*. Azaserine is a very potent inhibitor of these system
It inhibits the incorporation of glycine-2-C^{14} into cell purines by 90 per cen
or more at 0.10 μg. per reaction vessel *in vitro*, or 0.2 mg. per kg. body weigh
in vivo. The *in vivo* inhibition lasts 12 hours or more and does not affect th
incorporation in spleen at doses that are profoundly effective on the tumo

ells. No effect was observed on the incorporation of glycine-2-C^{14} into proein. The observed inhibition of *in vivo* purine incorporation correlates with creased survival time of azaserine-treated mice.

The *in vitro* system overcomes the inhibition in the later part of the incubaon in occasional experiments. This can be brought about predictably by lding supplements to the medium. Glutamine aids in overcoming the inibition, and boiled extracts of mouse or rat liver appear superior to glutamine.

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Discussion of the Paper

Doctor C. E. Wenner (*The Institute for Cancer Research and the Lankenau ospital Research Institute, Philadelphia, Pa.*): Doctor LePage has aptly illusated how ascites cell tumors can be used with facility for *in vitro* studies of icleic acid purine synthesis. The advantages offered by the ascites cells as escribed by Doctor LePage can also be applied to the evaluation of mechasms involved in the formation of intermediary carbohydrate metabolites r nucleic acid pentose synthesis.

The recognition of an additional pathway of glucose dissimilation *via* pentose as led to considerable interest in the extent to which this route participates the metabolism of glucose in cells. Since one of the obvious functions of is pathway is to supply pentose phosphate for incorporation into nucleic id, it was of particular interest to study the extent of this pathway in the etabolism of tumors and rapidly dividing cells. Although enzymes involved the hexosemonophosphate shunt had been demonstrated in neoplastic tises, this approach has been limited in that only maximum enzymic capacity determined and, in the intact cells, other factors would regulate the extent which this pathway is operative.

Table 1

Glucose Catabolism in Animal Tissue Slices

Tissue	No. of experiments	Per cent E. M.
t liver	5	46–99
t liver (fasted)	1	61
t liver (diabetic)	1	73
t liver (regenerated)	1	41 (56)*
ouse liver	2	27, 37
t heart, brain, kidney	1	100

* The values in parenthesis are calculated from experiments run simultaneously in which the incorporan of glucose-6-C^{14} into lactate was measured.

TABLE 2

ESTIMATION OF TRIOSE UNITS DERIVED VIA THE EMBDEN-MEYERHOF PATHWAY IN NEOPLASTIC TISSUE SLICES AND ASCITES CELLS

	Corrected RSA	Per cent of triose units derived via E.M.
Ascites		
Ehrlich carcinoma	1.0	100 (89)
Krebs-2 carcinoma	0.80	80
TA3 carcinoma	1.0	100 (97)
Tissue slices		
Rhabdomyosarcoma	0.91	91 (94)
Mammary adenocarcinoma	0.82	82 (87)

Recently, in the Weinhouse laboratories at the Institute, a method has bee employed that appears to provide a reliable estimation of the glucose catabol pathways in cells, *i.e.*, the extent to which the shunt pathway is operativ *versus* the classic Embden-Meyerhof symmetrical cleavage. Experimentally it involves the simultaneous oxidation of 1-C^{14}-labeled and uniformly C^{14} labeled glucose by aliquots of cells *in vitro* followed by isolation and C^{14} assa of a three carbon intermediate, such as lactate. By a comparison of the relative specific activity of lactate derived from glucose-1-C^{14} with that derive from U-C^{14} glucose, a value is obtained that is expressed as the corrected relative specific activity. From this, the percentage of lactate units derived vi the Embden-Meyerhof process can be calculated.

Data for normal tissue slices are given in TABLE 1. We need concern ourselves only with the last column, which gives the percentage of C-2 or C- units derived via the Embden-Meyerhof process. In the case of liver, wher lactic acid was not found to accumulate, it was found convenient to isolat acetoacetate produced by the condensation of 2-carbon units from which th pathways could be estimated by a modification of the calculations. It woul appear that, for the most part, the glycolytic pathway predominates in kidney heart, and brain. This is not the case, however, when liver slices are use since the shunt pathway appears to play an important part in glucose catabo

TABLE 3

EVALUATION OF GLUCOSE CATABOLIC PATHWAYS IN NEOPLASTIC TISSUE HOMOGENATES

Tissue	TPN	Corrected RSA	Per cent of trio units derived via E.M.
Hepatoma 98/15	—	1.0	100
Lettré-Ehrlich ascites	—	0.98	98
Lettré-Ehrlich ascites	0.002 *M*	0.86	86
Rhabdomyosarcoma	—	0.82	82
Rhabdomyosarcoma	0.0004 *M*	0.77	77
Rhabdomyosarcoma	0.002 *M*	0.64	64

ism. The data is indicative that the Embden-Meyerhof pathway is an im- ortant, although not an exclusive process, of glucose catabolism in liver. he variability of our data indicates to us, however, that the extent to which he alternate pathway is operative in liver is highly susceptible to factors that ave not been elucidated as yet.

In TABLE 2, quantitative evaluation is made of the Embden-Meyerhof path- vay in ascites cells and other neoplastic tissue slices. In all of the ascites umors studied, the Ehrlich carcinoma, Krebs-2 carcinoma, and TA3 adeno- arcinoma, practically all of the triose units are derived via the Embden-Meyer- of pathway. Hence it would appear that the shunt pathway does not con- ribute extensively to triose production from glucose. The solid tumor from vhich the TA3 ascites carcinoma was derived, the mammary adenocarcinoma, lso had little hexosemonophosphate shunt activity. Working with the ascites umor had an experimental advantage, inasmuch as more reproducible results re obtained since suspensions of these cells are easily pipetted. Also, the lifficulties associated with variability in the thickness of slices are absent. It ppears that for these tissues studied, this metabolic pattern of the ascites cell esembles the solid tumor from which it arose. Formation of triose in rhab- lomyosarcoma also resulted from catabolism of glucose via a symmetrical leavage, presumably the Embden-Meyerhof pathway. There appears to be resemblance among the neoplastic tissues studied here; that is, the Embden- Ieyerhof pathway is the major pathway for glucose catabolism.

It was of interest to evaluate the pathways for glucose catabolism in a omogenate system, where factors of cell structure do not come into play, and he relative capacity of the separate pathways for glucose oxidation may be nore readily determined than in the tissue slice. In addition, it was of interest o study the effect of cofactors on the extent of the pathways, which could not e carried out in the intact cell. Of especial importance is the coenzyme TPN, vhich is involved in the initial breakdown of glucose-6-phosphate via the exosemonophosphate shunt pathway. In TABLE 3, some preliminary experi- nents are reported. In the hepatoma 98/15 homogenate, no shunt pathway ppears to be operative. In the Lettré-Ehrlich ascites, TPN does not appear o alter the manner in which trioses are formed from glucose. There does ap- ear to be a slight effect of TPN in glucose cleavage in the rhabdomyosarcoma. categorical statement at this time, however, seems unwarranted.

In summary, this work should be considered as an initial attempt in the valuation of glucose catabolic pathways in animal tissues. For most normal nd all of the neoplastic tissues studied, trioses are largely derived via a sym- netrical cleavage of the glucose unit, presumably through the Embden-Meyer- of pathway. In view of the diverse pathways for pentose production, further ork will be necessary to trace the exact pathways by which the pentose and esoxypentose moieties of nucleic acid are formed.

ON THE CONTROL OF METABOLISM IN ASCITES TUMOR CELL SUSPENSIONS

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In spectroscopic studies of isolated rat liver mitochondria, it has been poss ble to identify different oxidation-reduction levels of the respiratory enzyme especially cytochrome *b* and dihydrode phosphopyridine nucleotide (DPNH depending upon the presence or absence of adenosine diphosphate (ADP), an to correlate these spectroscopically defined states with the acceleration inhibition of respiration caused by the addition or withdrawal of ADP fro such mitochondria.[1, 2]

Based on the idea that the glucose inhibition of respiration in ascites tum cells[3, 4, 5] may be related to the intracellular ADP level, we have applied th above spectroscopic test to such cells in the transition from respiration wit endogenous substrate to the glucose-inhibited state. Such spectroscop methods have already been used with ascites cells to record their cytochrom and pyridine nucleotide[7] content and to show by measurements of the rap kinetics of the components of their respiratory chain that capabilities for rap electron transport exist, but are not utilized.[8] In the current study, spectr scopic measurements of cytochrome *b* show definite changes in the intracellul ADP level caused by glucose addition. Other aspects of the glucose effect a revealed under our experimental conditions. The effect of glucose on respira tion is found to include an initial transitory phase of stimulation in which th ADP level is high. This phase is followed by a severely inhibitory phase which the ADP (or phosphate) level is too low to support appreciable respira tion. Measurements of glucose uptake show that it follows a similar cour of brief stimulation and then inhibition. These observations[9] allow us evaluate various theories for the Pasteur reaction and to suggest that it ma be a special case of extensive interactions of glycolytic and respiratory path ways in which exchange of adenosine polyphosphates between these two en zyme systems may be an important control mechanism*.[10]

Cell preparations and reaction media. The Ehrlich ascites tumor cells we prepared as before[7] but, in this case, special precautions were taken to avo appreciable changes of pH due to fermentative activity of the cells. Th Ringer solution was buffered with from 10 to 40 mM "tris" instead of pho phate. At the higher concentrations, the pH changed much less than 0 unit (~ 0.04) during the cycle of respiratory changes. In view of such a sma change and of the fact that addition of alkali did not reverse the inhibitio we may conclude that the effects recorded were not caused by a pH chang It was found that cells suspended in higher concentrations of "tris" buff deteriorated in several hours. Glucose concentrations used were about 20 mM, although our results are not critically affected by small changes of this value.

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* Doctor E. Racker, in discussing these experiments, was able to predict that a shuttle of ADP was occurri on the basis of the release of respiratory inhibition upon addition of methylene blue. Here our data in the tr sient stimulation and inhibition of both oxygen and glucose metabolism, together with our spectroscopic in cations of the intracellular ADP level, gives this theory direct experimental support.[10]

Method. Since the tumor-cell suspensions often contain clumps that settle to some extent during the period of spectroscopic observation, it is necessary to use a method that is rather insensitive to nonspecific optical effects, but which responds sensitively to the oxidation and reduction of the respiratory enzymes. For this purpose, we have used a double-beam spectrophotometer in which one beam has a wave length corresponding to a peak in the difference spectrum to be measured; the other beam, to a trough. An electronic circuit registers changes in the difference in light absorption at these two wavelengths, and is unresponsive to changes that are equal at both wavelengths.

Respiration is measured simultaneously by means of a vibrating platinum electrode that is inserted into the same cell suspension on which the spectroscopic measurements are made.[11]

The states of mitochondria. In order to justify the conclusions that we shall draw concerning the nature of the inhibitory reactions caused by glucose addition to ascites cells, we include TABLE 1, which describes the metabolic conditions characteristic of the various states of mitochondria, together with the oxidation-reduction levels of the respiratory components for the particular state.[4] In state 1, the endogenous substrate of the mitochondria gives a slow respiration that is limited by the intramitochondrial ADP level which, in turn, is set by the ATP-ase activity. Washed ascites tumor cell suspensions are in a similar state. When excess substrate and phosphate acceptor are added to the mitochondria, the actively respiring and phosphorylating state, 3, is obtained, and is terminated when the added ADP is phosphorylated and the inhibited or resting state, 4, is obtained.

Ascites tumor cells. A close analogy to the state 1-3-4 sequence of transitions is obtained when glucose is added to a suspension of ascites tumor cells, especially those prepared as described above. FIGURE 1 shows a combined record of respiration as measured by a vibrating platinum microelectrode, and the

TABLE 1

DEFINITIONS AND VALUES OF STEADY STATES IN RAT LIVER MITOCHONDRIA
SUBSTRATE: β-HYDROXYBUTYRATE; PHOSPHATE ACCEPTOR: ADENOSINE DIPHOSPHATE 25°

State	(O_2)	Characteristics				Steady state percentage reduction of components				
		ADP level	Substrate level	Respiration rate	Rate-limiting substance	a	c	b	Flavoprotein*	DPNH
1	>0	low	low	slow	ADP	0	7	17	21	~ 90
2	>0	high	~ 0	slow	substrate	0	0	0	0	0
3	>0	high	high	fast	respiratory chain	<4	6	16	20	53
4	>0	low	high	slow	ADP	0	14	35	40	>99
5	0	high	high	0	oxygen	100	100	100	150	100

* These values are based upon the amount of flavoprotein that is reduced upon addition of antimycin-A to the mitochondria in state 2, which is two thirds the state 5 value.

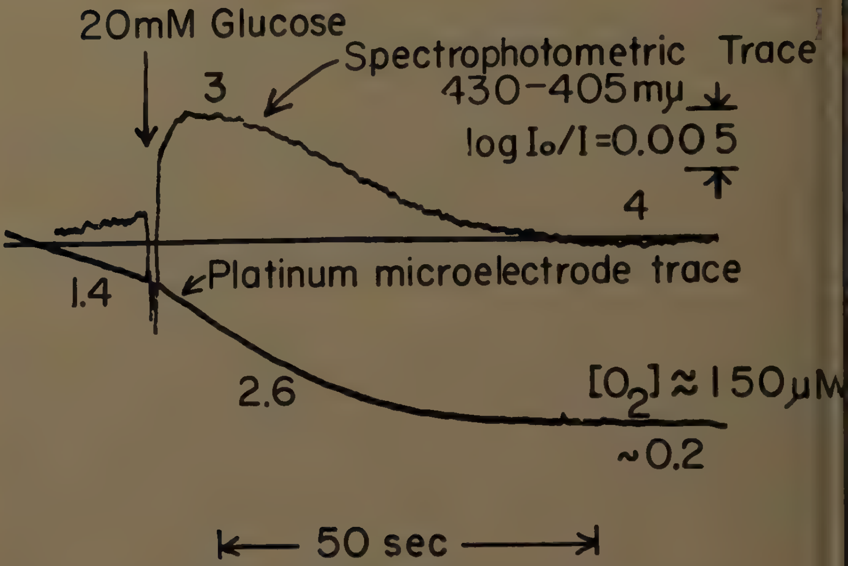

FIGURE 1. Simultaneous measurements of respiration and oxidation-reduction levels of cytochrome *b* up addition of glucose to a suspension of Ehrlich ascites tumor cells. Oxidation of cytochrome *b* is indicated an upward deflection of the trace. A decrease of oxygen concentration is registered as a downward deflectio (experiment 498d).

oxidation-reduction level of cytochrome *b* in the mitochondria of the ascite cells. The gentle downward slope of the respiration trace due to endogenou substrate and phosphate acceptor is greatly increased upon the addition glucose. The spectrophotometric trace registers an upward deflection tha corresponds to an optical density decrease at 430 mμ with respect to 405 m (an oxidation of reduced cytochrome *b*). Thus the two simultaneous respira tory and spectrophotometric events suggest a transition to state 3 from a stat closely approximating state 1 of the mitochondria. Other possibilities can b eliminated; for example, an increase of a DPN or cytochrome-linked substrat formed from glucose metabolism. Formation of such a substrate would caus a reduction of cytochrome, rather than the oxidation observed.

The other respiratory components that are affected in this transition cause by glucose addition have not been investigated in detail, partly because th optical density changes are hard to measure in turbid suspensions of ascite tumor cells. An oxidation of cytochrome *c* has been recorded and, in addition we have observed, interestingly enough, that DPNH is oxidized momentaril upon glucose addition provided the ascites cells are initially in the appropria state (FIGURE 2). While this result is in accord with the observations mitochondria, it is remarkable that the effect can be observed in the whole cells. An understanding of the effect can be obtained by a consideration of the dynamics of intracellular ADP.

The similarity of the effects recorded in whole cells and those observed with isolated mitochondria leads us to conclude that glucose addition causes a rise of the ADP level at the mitochondria of the intact cell. The obvious reaction for causing this increase is that catalyzed by hexokinase:

$$\text{glucose} + \text{ATP} \rightleftharpoons \text{glucose—6—PO}_4 + \text{ADP} \qquad (1)$$

The ATP would have accumulated in the cell prior to glucose addition. It is important to note that this reaction does not increase the intracellular phosphate level and, therefore, the respiration rate prior to glucose was limited by the ADP and not by the phosphate level.

That the ADP level should increase at the mitochondria and activate the respiratory chain in spite of the phosphorylation reactions in the glycolytic system; for example,

$$\text{1,3 diphosphoglyceric acid} + \text{ADP} \rightleftharpoons \text{ATP} + \text{3 phosphoglyceric acid} \qquad (2)$$

is understandable when the higher affinity of the respiratory chain for ADP[10] is considered. Of considerable interest is the fact that DPNH in the mitochondria is affected by the rise of ADP level prior to the reduction of DPN in the cytoplasm caused by the increase of the concentration of substrate for glyceraldehyde-3 phosphate dehydrogenase:

$$\begin{aligned}&\text{3-phosphoglyceraldehyde} + \text{DPN} + \text{phosphate} \rightleftharpoons \\ &\qquad\qquad \text{DPNH} + \text{H}^+ + \text{1,3 diphosphoglyceric acid} \qquad (3)\end{aligned}$$

Both these results can be explained if the ADP formed in the hexokinase reaction diffuses to the respiratory chain of the mitochondria before appreciable

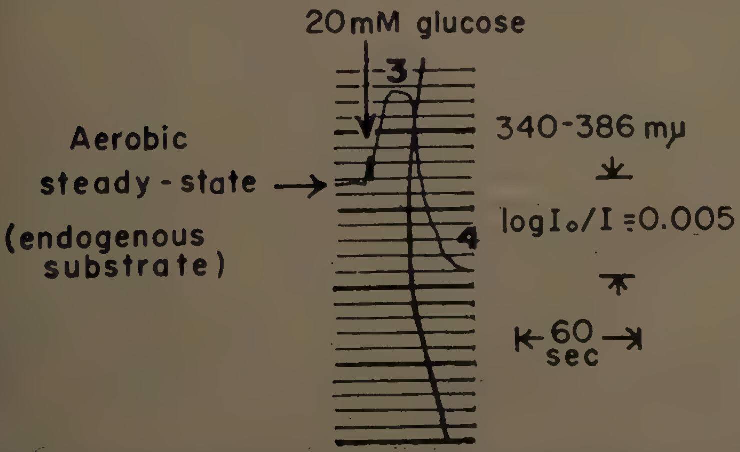

Figure 2. Kinetics of respiration-oxygen concentration as a function of time. Left-hand record shows a ypical acceleration and inhibition of respiration in an ascites tumor-cell suspension. In the right-hand record, he partial reversal of respiratory inhibition is caused by dicumarol addition (experiment 482b).

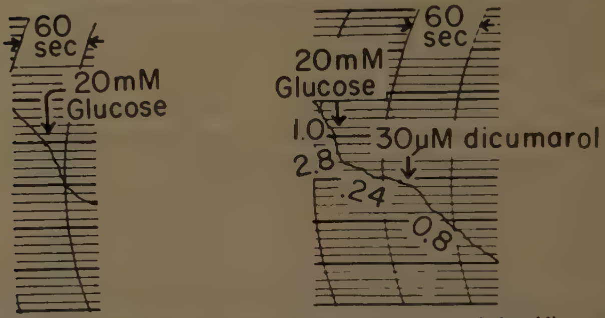

FIGURE 3. The effect of glucose addition upon the oxidation reduction level of pyridine nucleotides. An upward deflection indicates an oxidation (experiment 489b).

amounts of substrate are formed for either reduction of DPN or phosphorylation of ADP by the glycolytic system.

In isolated mitochondria, the phosphorylative activity characteristic state 3 is maintained for several hours if adequate phosphate acceptor is provided; for example, by hexokinase and glucose. But, in the ascites tumo suspension, state 3 lasts for only about a minute, and its termination is marke by an increased reduction of cytochrome *b* (a downward deflection of the trace and by a severe inhibition of respiration. The magnitude of this inhibitio under our experimental condition is remarkable. Over tenfold is recorded i FIGURE 1.

Confirmation of the idea that this inhibition is attributable to lack of pho phate or phosphate acceptor is afforded by the respiration records of FIGURE 3, which show that addition of the uncoupling agent, dicumarol, to the cel causes a partial recovery of respiration rate. Lack of substrate apparently not the cause of inhibition, since FIGURE 2 shows that the DPNH concentration is even higher in the inhibited cells than in the actively respiring ones.

A better understanding of the mechanism of this inhibition is afforded b FIGURE 4, in which analyses of the glucose levels are plotted as a function the time after glucose addition (these experiments were carried out by Doct Hess). It is clear that not only is respiration momentarily halted in the cells, but glucose metabolism is halted as well, and both processes can be restarted by addition of an uncoupling agent. It should be noted that th transient changes in both respiration and glucose metabolism are closely synchronized and that, according to EQUATION 1 above, the glucose metabolis directly determines the rate and amount of respiration. Thus measuremen of the changes in oxygen and glucose levels one minute after adding gluco should give the ADP/O value for the whole cell. Such studies are in progres

Discussion. Suspensions of ascites tumor cells provide a particularly interesting material for studies on the control of metabolism. In addition showing a pronounced inhibition of glycolysis by respiration (Pasteur effect they show the converse of this effect—an inverse Pasteur effect, the discover of which can be traced back to Crabtree in 1929.[3] Our spectrophotometri respiratory, and chemical data show new facets relevant to both the dire and the inverse effects that can be explained according to the hypotheses belo see also Chance, Hess, and Connelly[9]). The salient features of the results btained under our particular experimental conditions are that:

(1) A momentary stimulation of respiration precedes the inhibition reac-ion.

(2) The kinetics of glucose metabolism parallel those of oxygen.

(3) The inhibition is relieved by an uncoupling agent (*cf.* Chance and illiams[4]).

(4) There is a direct shuttle of ADP from the hexokinase system to the spiratory chain of the mitochondria (see footnote, p. 1008).

(5) The respiration rates of cells using endogenous substrate is limited by ie ADP level at the mitochondria (and not by phosphate). In the glucose-hibited cells, ADP or inorganic phosphate may be rate-limiting.

With these experimental data at hand, we are in a position to examine criti-ally older hypotheses, for example, those of Johnson[12] and Lynen,[13] and to ropose extensions and modifications thereof along lines suggested by Potter[9] nd also by Doctor Racker in his discussion of these results and in his own udies.[14]

At first we note that the very high affinity of the respiratory chain for ADP in accord with Johnson's requirements for an ADP-controlled Pasteur mech-

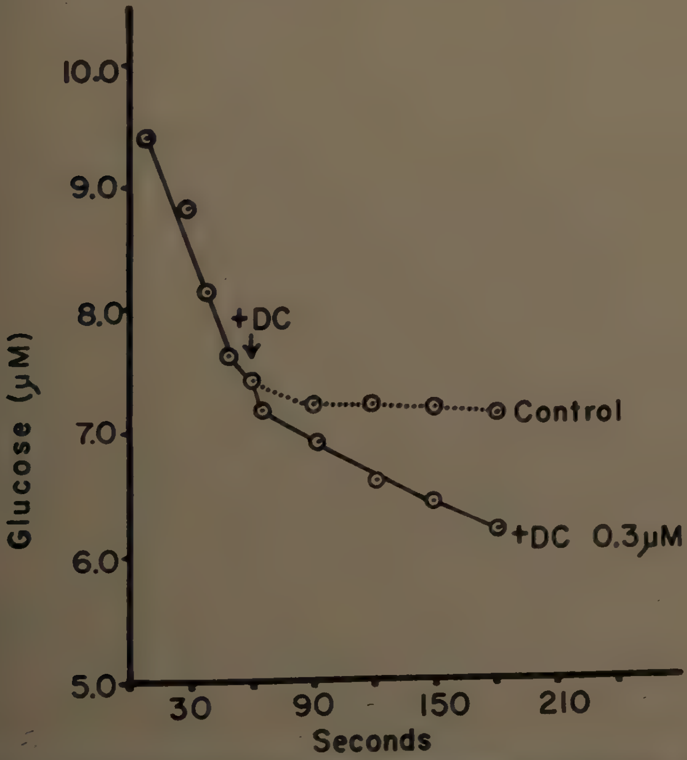

FIGURE 4. Glucose metabolism of ascites cells under the conditions of these experiments. Duplicate ex-riments, dicoumarol added to one. In this figure only, M is moles instead of moles per liter.

anism and, in addition, this reaction is practically irreversible in so far as th rate is not measurably diminished by a high ATP concentration. Our data however, show the need to consider not only the inhibition of glycolysis b respiration, but also the acceleration and inhibition of respiration upon addition of glucose to the ascites cells.

A common factor in this temporary inhibition of metabolism may be th adenosine polyphosphate level. Insufficient ADP is present in the mitochondria to permit ATP production, and insufficient ATP is present at hexokinase to permit glucose utilization. The affinity of the mitochondria fo ADP is sufficiently high[11] to compete successfully with the ADP dependen enzymes of glycolysis and thereby to cause their inhibition as well. A possibl cause for such an unbalanced distribution of the polyphosphates could be retention of ATP in the mitochondria. Such ATP could be made “unavailable either by specific binding or by a selective permeability of the membrane tha allows ADP to enter and prevents ATP from leaving during the special inhibiting phase of glucose metabolism that we have been studying. This latter proposal attaches special importance to the mitochondrial membrane in the regulation of metabolism, and surely a great variability of permeability of th membrane has been observed. The effects of phenolic uncoupling agents calcium, *etc.*, upon the mitochondrial permeability are very striking,[15] and othe agents or metabolic conditions could also vary their permeability, for exampl in the change from aerobiosis to anaerobiosis or in the presence or absence hormones.

The restarting of respiration upon addition of an uncoupling agent such a dicoumarol is similar to the “loss of respiratory control” observed when a uncoupling agent is added to liver mitochondria. The nearly simultaneou restarting of glucose utilization would require, according to this hypothesis, release of ATP stored in the mitochondria.* Glucose metabolism could the proceed continuously because the ability of the mitochondria to exhaust th intracellular ADP is inhibited by dicumarol.

An alternative hypothesis is that the initial phase of rapid respiration depletes the intracellular phosphate with a consequent inhibition of respiratio and ATP production at both the mitochondrial and glycolytic sites of phosphorylation. Addition of dicumarol to the phosphate-free system causes los of respiratory control (in spite of the lack of phosphate), breakdown of AT and release of ADP and inorganic phosphate to the glycolytic phosphorylatio sites. Difficulties of this hypothesis are (1) the relatively large concentratio of phosphate that is needed for the glycolytic phosphorylations and (2) phosphate added to the inhibited system does not start respiration.

A third hypothesis is based upon ATP inhibition of phosphohexokinas activity.[7] In this case, the respiratory activity caused by glucose additio raises the intracellular level of ATP to a point where phosphohexokinase activity is inhibited (this inhibition depends upon the ATP/Mg.$^{++}$ ratio;

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* Hydrolysis of intramitochondrial ATP, diffusion of ADP to the phosphorylation sites in the glycolytic sequence with ATP formation and with subsequent initiation of hexokinase activity, is a slower process and, ther fore, less likely to be an initial one.

value of 10 gives about 100 per cent inhibition*), and the accumulation of glucose-6-phosphate inhibits glucose uptake. An objection to this hypothesis is the fact that the glucose-glucose-6-phosphate equilibrium greatly favors the phosphorylated form, and a stoppage of glucose metabolism upon the utilization of only about one fifth of the added glucose (FIGURE 4) is improbable.

But the possibilities of product inhibition of hexokinase have been pointed out (for a discussion, see Racker[17]) and are not to be overlooked here. Purified yeast hexokinase does not show product inhibition; nevertheless yeast shows the Pasteur reaction. In addition, studies of yeast along the lines of this paper show a completely analogous, although less marked, series of changes in the oxygen and glucose metabolism, as well as in the oxidation-reduction levels of the cytochromes. Thus one must postulate an *in vivo* product inhibition of yeast hexokinase that has not been observed in studies of the purified enzyme.

Summary. In ascites tumor cells suspended in an appropriate medium, endogenous respiration is accelerated by glucose addition about twofold for approximately a minute, then an inhibition of both respiration and glucose metabolism occurs to an extent of 10- to 20-fold. Both the inhibitions are partially reversed by an uncoupling agent, dicumarol. Spectroscopic observations of the steady state oxidation-reduction levels of two components of the respiratory chain (cytochrome *b* and pyridine nucleotide) show, by analogy with studies of isolated liver mitochondria, that the initial, actively metabolising state is characterized by a high intracellular ADP level due to hexokinase activity, and the inhibitory phase by a very low level of either phosphate acceptor or phosphate. This phenomenon is a very direct representation of an inverse Pasteur effect, of the type suggested by Crabtree.[3] This inverse effect, the inhibition of respiration by glycolysis, occurs rapidly and is easier to study than the direct effect, the inhibition of glycolysis by respiration. And here the inverse effect can be shown to be directly related to the intracellular levels of phosphate acceptor or phosphate, as was postulated by the theories of Johnson[12] and Lynen[13] concerning the direct effect.

These results show that the variety of interactions of the enzymes of glycolysis and respiration extends well beyond the simple Pasteur reaction, which must now be regarded as a special case of generalized interactions that include various combinations of facilitations and inhibitions of one system by the other. The most likely of three proposed explanations for the generalized effect is based upon the ability of the mitochondria (1) to inhibit ADP dependent enzymes of glycolysis by depletion of the cytoplasmic ADP by virtue of the very high affinity of the mitochondria for ADP, and (2) to have a varying availability of ATP that depends upon the metabolic state of the mitochondria (aerobiosis and anaerobiosis) and upon the presence of uncoupling agents, and possibly of hormones as well.

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CARBOHYDRATE METABOLISM IN ASCITES TUMOR CELLS

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About six years ago, I stated in a lecture that carbohydrate catabolism is a ield in biochemistry in which we are on the solid ground of enzymology, and hat nearly all fundamental problems have been solved. What remained to be explored were problems of hormonal control and highly specialized problems of comparative carbohydrate biochemistry. This lecture, fortunately, was never published. It seems that during the past six years we have unlearned a great deal about carbohydrate metabolism, and I must admit today that our knowledge is so fragmentary that we have little accurate information about the fate of carbohydrates while and after they enter specific cells. What has brought about this change in attitude? The major reason is that we have come to recognize the existence of alternate pathways, and that we are just beginning to learn how to evaluate their relative quantitative significance.

The approach that Doctor Chance has just described represents a new phase in these studies. With ingenious methods of his own design he has studied metabolic events in intact cells and has analyzed electron transfer and phosphorylation processes in the steady state of intracellular metabolism. He has thus permitted us to glance behind the iron-porphyrin curtain of intracellular oxidations.

Another fruitful approach to the fate of carbohydrates in intact cells is being made with C^{14}-labeled glucose, which Doctor Wenner has discussed. I should ike to point out again the need for caution in the use of isotope incorporation studies for the quantitative evaluation of alternate pathways. Doctor Brown has made some very pertinent comments in regard to the occurrence of exhange reactions in nucleic acid synthesis. Since many biologists do not seem to be fully aware of the existence of these reactions, which may completely invalidate quantitative estimates of alternate pathways, or of the net synhesis of proteins, RNA or DNA deduced from incorporation studies only, I shall mention just one of many examples of exchange reactions. A rapid inorporation of $C^{14}O_2$ into oxalacetate is catalyzed by purified preparations of oxalacetate decarboxylase from *Micrococcus lysodeicticus*, although no net synthesis of oxalacetate from pyruvate and CO_2 can be detected. Thus, his process of isotope incorporation cannot be considered to be a synthetic pathway, in spite of the very rapid rate of labeling that takes place.

In our laboratory, Doctor Kvamme has studied the inhibitory effect of gluose on the respiration of Ehrlich ascites tumor, which was described by Kun *t al.*[1] and by McKee *et al.*[2] We have confirmed and extended some observaions made by these investigators. As shown in TABLE 1, it was found that sugars that serve as substrates for hexokinase (fructose, glucose, and mannose) nhibit oxygen uptake, while nonfermentable sugars (sucrose, ribose, and galacose) had no effect. An exploration of the concentration of glucose required

TABLE 1

INHIBITION OF THE RESPIRATION OF ASCITES TUMOR CELLS BY VARIOUS SUGARS

The test system contained in a final volume of 2 ml.: 50 μmoles of potassium phosphat
buffer pH 7.4, 160 μmoles of sodium chloride and a suspension of washed ascites cells (48
mg. protein) placed in the main compartment of a Warburg vessel. 150 μmoles of the sub
strate was placed in the side arm and tipped in after seven minutes of temperature equilibra
tion at 38°C.

Substrate	Cmm. O_2 after 30 minutes	pH at end of experiment
	217	7.50
Glucose	140	6.60
Mannose	133	6.60
Fructose	139	6.60
Sucrose	217	7.42*
Glycerol	210	7.50
Galactose	207	7.30*
Ribose	211	7.50

* Some glycolysis is most probably due to the presence of small amounts of glucose in the carbohydrates.

for the inhibition is shown in TABLE 2. At low concentrations, glucose usuall
stimulated somewhat, while, at high concentrations, O_2 uptake was markedl
impaired. Measurements of the pH at the end of these experiments showe
a marked drop in pH as shown in TABLE 2. An exploration of the oxidativ
capacity of extracts obtained from the tumor cells revealed a very diminishe
respiration below pH 7.0 with either succinate or α-ketoglutarate as substrat
Since, as shown above, in the presence of glucose, a pH of 6.5 or below can b
readily observed even in the extracellular suspension fluid, it is quite likel
that the rapid rate of aerobic glycolysis exhibited by these tumors increase
the intracellular hydrogen ion concentration and thus may lead to an impai
ment of respiration. It was concluded by McKee *et al.*,[2] however, and als
by Doctor Chance, that the inhibitory effect of glucose cannot be due entirel
to increase in hydrogen ions. Experiments with glycolytic inhibitors, such a
iodoacetate, which prevented the rapid pH drop without eliminating the in
hibitory effect of glucose, are in full agreement with this view (TABLE 3). I
is also apparent from these data that an alternate pathway for glucose is opera
tive in the ascites cells in the presence of IAA and methylene blue, since th
glycolytic pathway is nearly completely blocked under these conditions. Th
conclusion was supported by similar experiments carried out with cell-fre
extracts,* in which the rate of glucose oxidation was markedly stimulated b
the addition of triphosphopyridine nucleotide. The curious inhibitory effec
of methylene blue on endogenous respiration observed in experiments wit
intact cells (see TABLE 3) was explored further. As shown in TABLE 4, methy
ene blue inhibits pyruvate oxidation, as well as endogenous respiration, whi
it markedly stimulates respiration in the presence of glucose. Thus, it appea
that methylene blue has a dual effect, inhibiting mitochondrial oxidations vi
the Krebs cycle and accelerating the extramitochondrial oxidation of glucos
This hypothesis was verified in experiments with preparations of mitochondri

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* Doctor Kvamme has devised a mild and effective procedure for the preparation of cell-free extracts fro
ascites tumor cells that has been applied in our laboratory to other cells, including bacteria. The procedu
consists of shaking the cells with glass beads (0.1 mm. diameter) *in vacuo* in a cylindrical vessel.

TABLE 2

RELATION OF GLUCOSE CONCENTRATION TO INHIBITION OF TUMOR CELL RESPIRATION

Experimental conditions were as described in TABLE 1 except that fewer cells were used er reaction vessel (22.7 mg. protein) and the experiment was terminated after 60 minutes.

Substrate µmoles of glucose	Cmm. O_2	pH at end of experiment
—	225	7.6
1.2	236	7.52
2.4	247	7.45
4.8	220	7.32
7.2	162	6.98
9.8	116	6.69
12.0	122	6.60
76.8	122	6.49
192.0	112	6.43

nd supernatant fluid obtained from disrupted tumor cells. The stimulatory ffect of methylene blue on glucose oxidation in the intact cells may be due artly to its role as a hydrogen carrier and partly to its role as "uncoupler" f oxidative phosphorylation. Dinitrophenol, which is known to uncouple hosphorylation, also releases the inhibitory effect of glucose. This is in line vith the experiments of Kun *et al.*[1] and with Doctor Chance's observations vith dicoumarol. Methylene blue and dinitrophenol, however, not only unouple phosphorylation, but they also counteract the Pasteur effect. I shall ome back to this point later.

Finally, I should like to report some data Doctor Kvamme has obtained with ^{14}-labeled glucose. These experiments have revealed a peculiar regulatory unction exerted by the glucose concentration on the operation of alternate athways of glucose oxidation in ascites tumor cells. A comparison of the $^{14}O_2$ obtained from the oxidation of C-1- and C-6-labeled glucose showed that, t very low glucose concentrations, the oxidation of C-6 was equal, or slightly ess, than that of C-1. When the glucose concentrations were raised to a evel that inhibited endogenous respiration, $C^{14}O_2$ from C^{14}-1 glucose was

TABLE 3

EFFECTS OF SODIUM IODOACETATE (IAA) AND METHYLENE BLUE (MB) ON OXYGEN UPTAKE OF ASCITES TUMOR CELLS

The experimental conditions were as described in TABLE 1 except that fewer cells were sed (37.9 mg. protein per vessel). Equilibration at 38°C. was prolonged to 15 minutes to ermit the iodoacetate to act before the substrate was tipped in.

Additions	Cmm. O_2 after 30 minutes	pH at end of experiment
—	147	7.52
lucose (192 µmoles)	89	6.95
AA (0.14 µmoles)	138	7.52
AA + glucose	98	7.48
b (0.66 µmoles)	98	7.52
b + glucose	268	6.76
Mb + IAA	93	7.50
b + IAA + glucose	210	7.42

TABLE 4

EFFECT OF METHYLENE BLUE ON PYRUVATE AND GLUCOSE OXIDATION

Experimental conditions were as in TABLE 1.

Additions	Cmm. O_2	pH
—	187	7.55
Glucose (24 μmoles)	116	6.67
Mb (0.66 μmoles)	64	7.50
Glucose + Mb	180	6.55
Pyruvate (40 μmoles) + fumarate (4 μmoles)	270	7.55
Pyruvate + fumarate + Mb	75	7.52

about twice, from C^{14}-6 glucose less than half of that obtained at low glucos concentrations. It was thus apparent that high glucose concentration in hibited oxidation via the Krebs cycle, but increased the C-1 oxidation by th glucose-6-phosphate shunt. This stimulatory effect of high glucose concen tration was masked in the measurement of oxygen uptake because of the pro nounced inhibition of endogenous respiration. The ratio of C-1/C-6 oxidatio in ascites tumor cells is therefore variable and dependent on the concentratio of glucose. Dinitrophenol had only little effect on the oxidation of C-1 c glucose, but had a marked stimulatory effect on the oxidation of carbon 6 a high glucose concentrations.

I should like to propose here a unifying theory that would account for som of the data of Doctor Chance and of Doctor Kvamme, and that also offers a explanation for the Pasteur effect. This theory proposes that (1) the limitin factor in both extramitochondrial and intramitochondrial respiration is th concentration of adenine nucleotides; and that (2) shuttling takes place be tween intramitochondrial and extramitochondrial nucleotides, which is regu lated by the availability of substrates and oxygen, and is impaired in the pres ence of uncoupling agents. It can be visualized that during the oxidation c pyruvate the adenine nucleotides are held in the mitochondria, thus diminish ing their availability for extramitochondrial glucose phosphorylation. Ac cording to this mechanism, the Pasteur effect can be interpreted as an impair ment of the hexokinase reaction due to lack of adenine nucleotides at the sit of glucose phosphorylation. Dinitrophenol, which uncouples mitochondria phosphorylation should lead, according to this theory, to an increase in extra mitochondrial adenine nucleotides. These nucleotides, now available for th glycolytic process, accelerate glucose utilization and thus eliminate the Pasteu effect. On the other hand, an excessive demand on the adenine nucleotides b an active extramitochondrial system of glucose utilization as observed in thes tumor cells may be responsible for the inhibition of mitochondrial respiratio That adenosine triphosphate, formed during oxidative phosphorylation, i available for extramitochondrial systems is quite apparent from the fact tha glucose and hexokinase, added to respiring mitochondria, can serve as a suitabl acceptor system.*

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* Doctor Gatt has recently obtained in our laboratory more direct evidence for the validity of this theor In a reconstructed system consisting of liver mitochondria and the enzymes of glycolysis, a mutual inhibition respiration and glycolysis was observed. The inhibition was obtained only at low adenine nucleotide concen trations. Addition of 2,4-dinitrophenol released the inhibition of respiration and increased the production lactic acid.

In summary, these experiments serve to emphasize the difficulties encountered in an analysis of multiple pathways. When we measure glucose oxidation in ascites tumor cells, we are dealing with at least three different pathways localized in different intracellular structures: (1) the breakdown of glucose to triose phosphate, which is oxidized by an extramitochondrial oxidation system; (2) the extramitochondrial oxidation of glucose via a shunt pathway;* and (3) oxidation of lactic and pyruvic acids, the end products of glycolysis via the Krebs cycle in the mitochondria. In methylene blue, an agent was found that separates functionally extramitochondrial and intramitochondrial oxidation, the latter being inhibited, the former accelerated.

The inhibition of respiration by glucose, first observed by Crabtree in various tumors,[3] can be looked upon as an opposite to the Pasteur effect—the inhibition of glucose utilization by respiration. A hypothesis that assumes a shuttle of adenine nucleotides to and from the mitochondria may serve as an explanation for both these phenomena.

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* In the course of studies on the shunt pathway in ascites tumor, Doctor Wu has observed a rapid transformation of ribose-5-phosphate into hexose phosphates.

RESISTANCE TO COLCHICINE, TRYPAFLAVINE, AND RADIATION IN SELECTED SUBLINES OF THE EHRLICH ASCITES TUMOR

By Hans Lettré

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In 1939, we began an investigation of the actions of chemical compounds o normal and malignant cells grown *in vitro*.[1] Experiments with tissue culture enable us to bring the chemical factors in direct contact with the cells and th concentration of the drug used is limited only by its solubility in the medium *In vivo*, other factors interfere, however, *e.g.*, the toxicity of the compoun that may prohibit using a dosage needed for an action on the cells. Furthermore, in experiments with solid tumors, the contact of the compound with th tumor cells is dependent on its distribution in the body fluids and its abilit to penetrate to the tumor cells. Therefore, I thought it suitable to make us of a form of tumor growth that is similar to the conditions of the tissue cultur and studied the behavior of an ascites tumor (Ehrlich mouse ascites tumor under the influence of chemical factors.[1,2]

Comparing tissue culture, ascites tumors, and solid tumors, there are th following differences characteristic for each system in a screening procedure:

(1) In tissue culture, a compound is in direct contact with the cells, and it concentration is constant during its time of action. A change in the concentration occurs only with labile compounds or by an interaction with the cells.

(2) In ascites tumors, the compound can be brought by intraperitoneal injection into direct contact with the tumor cells. In contrast to the tissue culture experiments, however, excretion does not allow the concentration t remain constant during a long time, and it decreases according to the propertie of the compound.

(3) In solid tumors, the concentration of the compound depends on man factors, if we avoid intratumoral injection. The properties of the compound as well as those of the tumor, will be responsible for the concentration of th compound in the tumor which, usually, will first increase and later decrease.

As far as the author knows, the first ascites tumor was described in 1927 b Hesse[3] in the case of the Flexner-Jobling tumor of the rat. In 1932, Loewenthal and Jahn[4] reported the transformation of the Ehrlich mouse carcinoma i an ascites form. These authors and Collier and Jahn[5] claimed this tumor t be chemoresistant and, consequently, they considered a virus to be responsibl for the malignancy. As we showed,[6] the cells of the ascites tumor lose thei transplantability by homogenization. Thus, this assumption cannot be valid Furthermore, we could show that the ascites tumor is an extremely sensitiv indicator for the action of a suitable chemical factor.

A few days after the inoculation of 0.2 ml. of ascites from a tumor mouse, th weight of the injected animal increases (up to 20 g. in 10 days), and the weigh curve serves as an indicator for the development of the tumor. The surviva time of the animals is between 12 to 14 days on the average. The single an als show differences in the weight curves but, using the average weight of roups of five animals (comparable in provenance and starting weight and un- er similar conditions), a comparison with a group of five other comparable ice is possible. Therefore, for the testing of a compound, the following ethod was used. From three groups of five mice, the first is inoculated only ith 0.2 ml. of the same tumor ascites that is also used for the other groups. he second group receives the ascites and, in addition, the compound in solu- ion on the same day and the following four days, so that no more than a total f 0.5 ml. are injected daily. As the development of the ascites tumor has ad- anced in five days, as in the case of no injection or the use of an ineffective com- ound, therefore we stop the injections at this time. The third group receives nly the solution of the compound during five days. The minimal effective osage can be estimated by variation of the dosage of a compound. Criteria or the effect with this method are: (1) the comparison of the weight curve of he treated group with that of the group with tumor alone; (2) the survival ime between the groups. A difference in the weight curves alone, *e.g.*, with oxic dosages, does not indicate a positive effect. Only when this occurs to- ether with an increase in the survival time is the effect worthwhile. With the uppression of the development of an ascites tumor, we often observe the growth f a solid tumor that would not otherwise have come to the fore during the rapid rowth of the ascites tumor.

With colchicine as an example, we observed a remarkable difference in the eight curves under a daily dosage of 10 μg. and an increase of the 50 per cent urvival time from 14 to 28 days. We used this method in 400 experiments etween 1940 and 1942 and, in the six years since 1949, we have used it in ,000 additional experiments.[8, 9, 10] During the last few years, we have used he Ehrlich ascites tumor for the study of the effect of repeated action of exog- nous factors.

In the experiments with colchicine, only 10 per cent of the mice could be rotected against the Ehrlich ascites tumor. In the literature, there are only hree examples of a complete suppression of animal tumors by colchichine: the hope papilloma of the rabbit (Peyron, Lafay, and Kobozieff[11]); a lympho- arcoma of the mouse (Bass and Probert[12]); and a rat sarcoma (Bloch-Franken- hal and Back[13]). We were able to suppress the growth of the Yoshida tumor f the rat completely with a derivative of colchicine, the N-Methyl-colchicamide NMC) (see below), but not with colchicine itself (unpublished). The differ- nce in the response of different tumors may be caused by a different sensitivity f the tumors or, in these cases of grafted tumors, by the host-tumor relation hich, by genetic differences, would support the suppression of the tumor. urthermore, the tumor cells could be a mixed population of cells with different ensitivity, and the tumor growth after treatment could be caused by less ensitive cells selected under the action of the agent. This selection of resistant ells was demonstrated by Law[14] with strains of mouse leukemia under the ction of antifolic acid derivatives. Another example is shown in the experi- ents of Jackson[15] in the case of the Walker carcinoma of the rat under treat- ent with triethylenemelamine.

In the case of a mitotic poison, such as colchicine, with a specific action on cells only in the stage of mitosis, one has to investigate the question whether an inhibition of mitosis of all cells is possible or whether the heretofore needed dosage would hinder part of the cells entering mitosis. Brodersen[16] in 1942 analyzed the action of some compounds on the cell division of the cells of an ascites tumor. After the injection of a compound into an animal with ascites tumor, one can make a puncture from time to time and, from the stained smear preparations, follow up the action of the compound.

Using this method, we could demonstrate the increase of activity of NMC in comparison with colchicine.[17, 18] After studying the effect of a single injection of colchicine or NMC we compared the action of fractionating a given dosage.[19] We found, for instance, that the single injection of 20 μg. NMC caused a smaller effect than the injection of 2 doses of 10 μg. or that of 6 doses of 3 μg. By varying each dose and the sequence of the injections, we tried to achieve a further increase of the amount of arrested mitosis. The strongest effect ever reached, however, was not more than 95 per cent, and we have not attained a 100 per cent one. From this, it is possible to conclude that the nature of the process makes it fundamentally impossible to arrest every cell in the state of mitosis, or that this tumor contains cells of less sensitivity.

Therefore, we have performed a series of transplantations of the ascites tumor under repeated injection of these compounds.[20, 21] As soon after a five-day treatment as an amount of ascites tumor just sufficient for the next transfer had been developed, a new group of mice was inoculated with this tumor, and the treatment was repeated. This procedure was continued as long as possible. In 9 of 10 independent series of this type, the tumor died out in the sixth or seventh transfer. These results would show, therefore, that, independently of the morphologically visible effect, all cells under this repeated treatment were injured to a degree of complete loss of virulence. But in one of these 10 experiments, there was noticeable, as early as the fifth transfer, a rapid growth of the tumor even under the injection of NMC and in the following transfers. This tumor was no longer inhibited by colchicine or NMC. The cells of this subline of the Ehrlich mouse ascites tumor divide by mitosis and contain a spindle. From this subline in the 24th transfer, we branched off and bred another subline, which was no longer treated with the mitotic poisons. From every 10th transfer, we tested the resistance against colchicine or NMC. The resistance was retained to the 80th transfer. Later, this line, bred without further injections, died out for unknown reasons. The main line of this resistant tumor which continued to be treated with the five-day injections of 3 μg. NMC is now (since 1951) in the 188th transfer and proves to be extraordinarily virulent. The survival time of the animals after the inoculation of this tumor line is a 9-day average, as against 14 days for the original Ehrlich ascites tumor.

According to the findings of Law, the development of resistant strains is the result of spontaneous mutation with selection by means of the agent. During the last few years, we performed transplantations of single cells of our hyperdiploid ascites tumor. In 10 of 367 transplantations, H. Querner[22] obtained ones, which we have examined for their sensitivity against colchicine. Only ne clone showed a reduced sensitivity. Therefore, it must be suspected that iere were resistant cells in the original cell population.

The colchicine resistant subline proved not to be generally chemoresistant. ike the original tumor, it is inhibited by other compounds such as nitrogen iustard or patuline. Toward acriflavine, it proved to be less sensitive but not illy resistant.

Concerning the mechanism of the colchicine action in the inhibition of the rowth of a sensitive tumor, the existence of this resistant strain is important. udford believed the colchicine action to be an indirect one, *e.g.*, by injury of he capillaries. But since the host gets the same degree of injury with the esistant subline, the colchicine action must be a direct one.

A natural resistance of the golden hamster against colchicine has been estab- shed by Orsini and Pansky.[23] Adler and Meerovich[24] produced a colchicine esistant strain of *Trichomonos vaginalis*. In the plant kingdom, the natural esistance of *Colchicum autumnale* against its own alkaloid is known.[25] Also, east, which can be polyploidized with acenaphthene, for example, does not espond to colchicine.[25]

Experiments of the same type have also been performed with other sub- tances. Using patulin in all series thus far performed, the tumor died out in he sixth to eighth transfer. Aminopterin has a strong inhibitory activity pon the development of the ascites tumor but, especially with this compound, ne can observe the growth of solid tumors. We never observed a resistance f the ascites tumor against Aminopterin. The sensitivity remained un- hanged. Likewise experiments with nitrogen mustard and with actinomycin id not lead to resistant lines. These negative results would not exclude the ossibility that resistant strains could exist. It is still an open question whether very tumor type is able to develop resistance against every compound, or hether every special tumor type has only a limited number of possibilities of esistance corresponding to its specific biochemical pattern.

Using acriflavine, we had, in one experimental series, no changes of sensi- ivity after 25 transfers. In another series, we obtained a decrease of sensi- ivity which does not have, as yet, the characteristics of full resistance. This ubline is under further investigation.

In addition to these investigations with chemical factors, two other examples f steady influence of exogenous factors can be mentioned. The first is an ttempt to develop a resistance to X rays performed by G. Schubert at Ham- urg, Germany.[26] The hyperdiploid Ehrlich mouse ascites tumor was injected ntramuscularly in both rear thighs. Only the solid tumor on one side was rradiated. The irradiated tumor was transplanted and again irradiated ac- ording to this scheme. This was continued, and less sensitive tumors were btained, which could be changed back to the ascites form. This ascites form as bred for one year in our institute, and it proved itself to be sensitive against olchicine, NMC, acriflavine, patuline, and nitrogen mustard. After retrans- ormation to a solid tumor form, this line showed still less sensitivity against irradiation in comparison with the growth of the original tumor under the sam conditions. There is also a lessening of mitotic disturbances after irradiatio in this line.

Another example of a steady influence of an outside factor on tumor growt are our experiments with animals that are kept at raised temperature (38° C. or daily subjected to 41° C. for a short time.[21, 27] Such a line has been mai tained since October 1951. There results a slowing of the growth speed of th tumors during several transfers that leads after a time to a constant characte Though, in this case, we do not get real resistant forms, this method should b mentioned as a possibility for gaining substrains with altered qualities.

The problem of resistance of tumors against exogenous factors seems to b one of the most important in the field of tumor therapy. Many observation made thus far lead to the conclusion that, in principle, every tumor form ha the ability to develop a resistant form against every agent applied, and tha chemotherapeutical agents bring these resistant forms to the fore. Therefor we should make more use of such resistant lines in experimental chemotherapeutical studies. The aim of the investigations described here is the esta lishment of a spectrum of tumors characterized by their sensitivity or resistanc against a number of exogenous factors. Such a tumor spectrum should be greatest value for further studies of new tumor-inhibiting agents.

Loewenthal and Jahn are to be thanked for the development of the ascite form of the Ehrlich mouse carcinoma, but their claim of a general chemoresis ance of this tumor has hindered its use for screening studies. In discussing th problem of chemoresistance today, it is noteworthy that the point of view ha now changed in favor of specific chemoresistance, its explanation and conques

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CONCLUDING REMARKS

By Alexander Haddow

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I have been asked to summarize what has been an intensive program. It a difficult task—indeed scarcely possible—but the honor is one that I greatl appreciate. I can only put forward various reflections. The whole history biology and medicine has been repeatedly influenced by unique technic developments such as, for example, the origination of the nutrient agar plat the propagation of bacteria that it allowed, and techniques for the cultivatio or maintenance of various protozoa, including especially the trypanosome All these were the signal for great advances. So, too, there have been gre phases in cancer research: the discovery at the end of the 19th century of th intraspecific transplantability of tumors; the great work on the fundament biological properties of the cancer cell, carried out at the Imperial Cance Research Fund, London, in the early years of the present century by Bashfor Murray, Woglom, and others; and the demonstration by Tsutsui, in 1915, the susceptibility of mouse skin to the induction of tumors with coal tar. Wit all these must rank the recognition and study of the ascites tumor. As Doct Klein has made clear, this was, in fact, a rediscovery, based upon the observa tion of Hesse, in 1927, that the Flexner-Jobling rat carcinoma can be tran mitted by the ascitic fluid that develops during the growth of this tumor i the peritoneal cavity and, upon the confirmation of this finding by Koch, wh described how this method of transmission resulted in an increased percenta of takes when compared with subcutaneous transplantation. In his exper ments, Koch was endeavoring to prove an assumed protozoan etiology cancer. If many of his arguments no longer apply, his findings remain interest, particularly his description of gradually increasing cellularity of th ascitic fluid with time, the preservation of transmissibility on cold storage, th loss of activity on heating and after filtration, and the technique wherewith t obtain normal peritoneal fluid from healthy rats for comparative studies. I was Koch's collaborators, Loewenthal and Jahn, who, after many unsuccessf attempts, eventually succeeded, in 1932—a generation ago—in obtaining new transfer line of the Ehrlich carcinoma, carried by serial intraperitone passage of peritoneal exudates, and called it an "ascites tumor."

This leads me to digress on whether it would not be worthwhile to set on foo a more systematic survey of the older literature of cancer, over the past five six decades, since many an observation must have been made, of little or un recognized interest at the time, but that might now have the greatest im portance in the light of more recent developments. We have seen a recen example in the rediscovery of antigen-antibody reactions in gels, based upo older observations, and now developed to such great effect by Ouchterlon Bjørklund, Grabar, and others.

The techniques underlying the ascites tumor, as we have seen, have allowe greater precision quantitatively. They point, as has been said, to a ne genetics of somatic tissues, have advantages in the study of cellular metabolism nd of chemotherapy, permit substantial advantages in cytology through the vailability of tumor cell suspensions in nearly pure culture, and derive much rom the analogies drawn by Lederberg and Patt with the phases of growth of microorganisms. Other advantages lie in the possibilities for more detailed knowledge of the chromosome complex, both numerically and structurally, as has been shown by Selby, Biesele, and Grey, and by Dalton, by Levan, by Gey; by Wilton Earle in the feasibility of production of large volumes of specific umor cells; by others, for the biochemical comparison of the normal and the malignant cell; and by Yoshida's successful demonstration of transplantation of the single cell.

Doctor Klein has provided us with an outstanding account of the principles underlying the phenomenon of the convertibility of solid tumors into the ascites orm, gradually, immediately, or not at all. And we must be specially grateful or the collaboration of the past few years between himself in Stockholm, Levan in Lund, and Hauschka in the United States. As far as Hauschka's formidable contribution is concerned, it should be made clear that simple duplication of the chromosome set, as such, is not the essential factor in loss of antigenicity, nor in the genotypic change that leads to increased malignancy. Most diploid tumors have a few balanced polyploids with no immunoselective value. Variability within such balanced polyploids, however, eventually leads to heteroploidy, and it is these cells that have greater resistance to all kinds of unfavorable environments, including immunologically hostile or refractory environments. For example, Koller has shown that they are more radioresistant, Yoshida has shown that they are more resistant to the action of nitrogen mustard, and McCardle has shown that they are more resistant to the action of podophyllotoxin. Sparrow and others have referred to the general question of radioresistance and polyploidy and, clearly, there is need for much more work. In this connection, Koller reminds me of Muller's demonstration that a triploid *Drosophila* was no more and no less resistant than the diploid. On the other hand, in Sax's comparison of diploid and tetraploid *Tradescantia*, the tetraploid was the more radioresistant.

Two other points occur to me as of special interest. First, the brief mention of an observation by Doctor and Mrs. Klein, of the mutual depression of two tumors growing intraperitoneally. One has often conjectured whether two tumors, carrying separate deficiencies, might repair each other by genetical nterchange or recombination. Here is a problem that could be challenged deally by the use of ascites tumors. Secondly, Doctor Levan mentioned a peculiar ring chromosome propagating in the stem line, and recalled Koller's description, in 1953, of similar anomalies in tumors induced by various nitrogen mustards, and propagating, as it were, an imprint of the carcinogen that induced their appearance Although he is a member of my own group, you will perhaps allow me to congratulate Koller on his study of human carcinomatosis, with its indications—albeit with exceptions—of the well-differentiated colony-forming adenocarcinoma on the one hand, and of the anaplastic free cell type on the other.

Last, but by no means least, are Leuchtenberger's parallelism between DN concentration and chromosome number, still requiring further study in th case of tumors, and the excellence of Richard's account of the measurement c chromosome numbers by chemical means and his study of the variability c DNA according to chromosome number, polyploidy, polyteny, DNA synthesi and errors in measurement, even though the time of maximal synthesis in th cell cycle is still unclear, and must be one of the main subjects for attack in th next few years.

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